IN VITRO MORPHOGENESIS AND INHERITANCE OF IN VITRO TRAITS IN DESMODIUM

By

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Desmodium, *Desmodium sp.*, is a forage legume widely cultivated in the tropics and of growing importance in the southern United States. Previous work aimed at application of biotechnological methods to this crop had obtained limited in vitro regeneration from seedling hypocotyl explants in a single genotype. The objective of the present work was to examine the potential for improving regeneration response in desmodium through optimization of culture protocols and through genetic improvement.

Efforts at identifying alternative explant sources focused on seedling cotyledons, mature leaf disks, and mature petioles. Regeneration could not be induced from any of these explant types. Several combinations of 2,4-D and kinetin concentrations in the shoot bud induction medium were examined in an unsuccessful effort to obtain regeneration from hypocotyl explants of recalcitrant genotypes. Direct regeneration from hypocotyl explants without an intervening callus growth step was also unsuccessful. Several treatments were investigated with the aim of enhancing regeneration response in the previously identified regenerating genotype. The best protocol identified consisted of a

28-day callus induction step with 0.06 mg L^{-1} picloram (4-amino-3,5,6-trichloropicolinic acid) and 0.1 mg L^{-1} BA (6-benzylaminopurine), a 14-day shoot bud induction step with 1.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} adenine, and a 28-day shoot elongation step with 0.012 mg L^{-1} picloram and 0.2 mg L^{-1} BA. Under this protocol, shoot production occurred in 63% of explants.

Extensive efforts at hybridizing regenerating and nonregenerating genotypes yielded two crosses. Callus growth and regeneration capacity were evaluated in the parental lines, the F_2 , and F_3 generations. Based on joint scaling tests and variance partitioning, neither trait was found to fit a simple additive-dominant genetic model. Both traits were moderately to highly heritable, as determined by parent-offspring regression. Heritability of callus growth ranged from 0.52 to 0.77, depending on the cross and on the numerical scale employed. Heritability of the regeneration trait ranged from 0.14 to 0.46. Members of two F_3 families exhibited much more vigorous and prolific regeneration than the regenerating parental genotype.

CHAPTER 1
INTRODUCTION

The last two decades have seen a surge of interest in biodiversity within the agricultural research community. In order to exploit diverse agricultural environments in an efficient, environmentally benign manner, the full range of available genetic resources must be examined and utilized. Extensive research effort is aimed at developing new crops and identifying new regions of application for existing crops. Desmodium (*Desmodium* spp.) is one of several exotic forage legumes that show potential for use in the pastures of Florida and the adjacent southeastern United States.

Desmodium is a large genus consisting primarily of perennial herbs and shrubs. The genus is distributed in tropical and subtropical regions worldwide, with a probable center of origin in Southeast Asia and a secondary center of diversity in Mexico (Ohashi, 1973; Schubert, 1963). Classification within this genus is difficult due to continuity of taxonomic characters among species, and estimates of the number of species range from 200 (Takahashi, 1952) to 500 (Younge et al., 1964). Most of the species studied have chromosome numbers of 2n=2x=22, and are predominantly self-pollinated (Chow and Crowder, 1972, 1973; Rotar and Uruta, 1967).

The agronomic role of the genus *Desmodium* has been reviewed by Imrie et al. (1983). Several desmodium species are grown in significant acreages in tropical or subtropical pastures. *D. intortum* (Mill.) Urb. (greenleaf desmodium) and *D. uncinatum* (Jacq.) DC. (silverleaf desmodium) are widely used in humid subtropical regions of Australia, Africa, and South America.

D. heterocarpon (L.) DC. (carpon desmodium) has been released as the cultivar 'Florida' for pastures in the southeastern United States (Kretchmer et al., 1976). This species flowers earlier in the fall than either greenleaf or silverleaf, and is thus adapted to areas where occasional early frosts threaten seed production. *D. ovalifolium* Guill and Perr. is grown in Southeast Asia and has attracted attention elsewhere for its tolerance to acid, low fertility soils and shaded conditions (Schultz-Kraft and Pattanavibul, 1985). Other desmodium species with agronomic potential include *D. heterophyllum* (Willd.) DC., *D. sandwicense* E. May., and *D. barbatum* Benth.

Most desmodium cultivars are simply selections from germplasm collections, and genetic improvement of this crop has received minimal attention until the last decade. A desmodium breeding program was initiated at the University of Florida in the late 1980's, with the primary breeding objectives of improved forage quality, rapid establishment, increased seed production, and resistance to root knot nematodes. The University of Florida program utilizes germplasm from *Desmodium heterocarpon* ssp. *heterocarpon*, *D. heterocarpon* ssp. *angustifolium* (Craig) Ohashi, and *D. ovalifolium*. The species *heterocarpon* and *ovalifolium* are closely related and yield fertile progeny upon intercrossing (Quesenberry et al., 1989). Some authors include the species *ovalifolium* within species *heterocarpon* (Ohashi, 1983), but the two are morphologically distinct and are generally regarded as separate entities by agronomic researchers (Imrie et al., 1983; Schultze-Kraft and Benavides, 1988). *D. heterocarpon* produces elongated inflorescences and glabrous to slightly pubescent seed pods, while *D. ovalifolium* has compact inflorescences and bears heavily pubescent pods. Leaves are opaque in *ovalifolium*, glabrous to slightly pubescent in *heterocarpon* ssp. *heterocarpon*, and coriacious in *heterocarpon* ssp. *angustifolium*. Subspecies *angustifolium* is further

distinguished by a leaf length/width ratio of 3.0 or greater, as opposed to 1.0 to 2.5 in ssp. *heterocarpon* and *D. ovalifolium* (Quesenberry et al., 1989), and by a more erect growth habit.

Recent advances in biotechnology have extended the potential for genetic improvement of both new and established crop species. Plant breeding has traditionally relied upon genetic variation existing within the species of interest or in closely related, sexually compatible species. Current genetic transformation methods allow desirable genes to be moved between distantly related or nonrelated species, thereby greatly expanding the potential gene pool for any specific crop (Fraley et al., 1986). Among forage legumes, *Agrobacterium*-mediated genetic transformation has been used to insert reporter genes into alfalfa (Deak et al., 1986), white clover (White and Greenwood, 1987), and red clover (Quesenberry et al., 1992), and *Stylosanthes* (Sarria et al. 1994). Future application of transformation techniques to forage legumes may involve introduction of specific genes for pathogen resistance, herbicide resistance, or forage quality.

Because genetic transformation occurs on a single cell basis, obtaining transformed plants requires that whole plants be regenerated from single cells, or from small cell aggregates. A protocol for efficient in vitro plantlet regeneration is therefore highly desirable for transformation work. Besides serving as a tool for genetic transformation, cell and tissue culture methodologies can be useful for in vitro screening of germplasm for resistance to pathogens, herbicides, and environmental stresses (Hughes, 1983).

Published work on the in vitro culture of desmodium is sparse. Angeloni et al. (1988) regenerated *D. affine* and *D. incanum* from shoot tip cultures using a modified MS medium (Murashige and Skoog, 1962), but failed to obtain regeneration from leaf or anther explants. Using seedling hypocotyl explants

from six diverse desmodium genotypes, Wofford et al. (1992b) evaluated two tissue culture protocols that had been previously demonstrated effective for legumes. Regeneration was only observed in a single genotype of *D. heterocarpon* ssp. *angustifolium*, using an L2 based protocol originally developed for use with *Trifolium* (Phillips and Collins, 1980).

If in vitro techniques are to be most effectively employed for the genetic improvement of desmodium, it will first be necessary to broaden the range of regenerating genotypes. This may be accomplished either by modification of the culture protocol or by breeding for regeneration ability. Nonregenerating genotypes can often be induced to regenerate by manipulating growth regulator levels or any of several other culture parameters. In many species, however, the majority of genotypes have resisted exhaustive efforts to induce regeneration (Flick et al., 1983; Ammirato, 1983). Breeding for regeneration ability has been effective for other forage crop species, including alfalfa (Reisch and Bingham, 1980) and red clover (Quesenberry et al. 1992). The efficiency of such a breeding effort is dependent on the genetic basis of the regeneration trait. Investigations of the inheritance of this trait in several higher plant species has shown that the trait may be either qualitatively or quantitatively controlled, and can be subject to cytoplasmic effects (Keyes et al., 1980; Kumar et al., 1985; Wan et al., 1988).

The remainder of this dissertation addresses both of these approaches to broadening the range of regenerable desmodium lines. Chapter 2 examines a variety of culture protocols with the objectives of inducing regeneration in a wider range of genotypes and optimizing the response of established regenerator genotypes. In Chapter 3 the genetic basis of callus growth and regeneration is examined through the production of hybrids between regenerating and nonregenerating lines and evaluation of the resulting F_2 and

F_3 generations. Chapter 4 presents a summary and conclusions, and discusses the implications of this work within the broad context of genotype by environment interaction under in vitro conditions.

CHAPTER 2
OPTIMIZATION OF IN VITRO REGENERATION PROTOCOL

Although numerous members of the family Leguminoseae have been regenerated in vitro, the family is regarded to be difficult with respect to in vitro regeneration (Flick et al., 1983). Regeneration frequency is low for many species, and specific culture requirements can vary widely both among and within species (Phillips and Collins, 1983).

Regeneration in angiosperms can be accomplished via either of two conceptually distinct pathways—organogenesis or somatic embryogenesis. Both types of regeneration can be induced either directly from the initial explant source, or from callus or suspension cells generated in culture. Organogenesis usually involves production of a shoot meristem followed by shoot elongation and rooting. Shoot and root regeneration are discrete processes occurring in response to specific culture conditions, particularly the types and concentrations of plant growth regulators in the medium. In somatic embryogenesis, shoot and root meristems are produced simultaneously in a process similar to zygotic embryo development. Maturation and germination of somatic embryos can be induced by manipulating plant growth regulator levels, or may occur spontaneously under constant culture conditions (Hazra et al., 1989).

In practice, the distinction between organogenesis and embryogenesis is not always so clear (Ammirato, 1983). Ideally, somatic embryos should closely resemble sexual embryos and possess clearly identifiable shoot, root, and cotyledonary primordia that subsequently develop into their respective organs. Deviations from this ideal situation include abnormal or absent cotyledons and

failure of the radicle to produce a root (Tisserat et al., 1978; Sellars et al., 1990). The situation can be further complicated by complete failure of the somatic embryo to germinate, sometimes followed by adventitious bud production from embryo tissue (Saunders et al., 1987; Vasil, 1987). Due to the frequent occurrence of these and other developmental abnormalities during somatic embryogenesis, histological examination is necessary to conclusively distinguish between embryogenesis and organogenesis. Somatic embryos are characterized by two critical features: a bipolar morphology with discrete coleoptile and coleorhiza, and a lack of vascular connections to the surrounding tissue (Haccuis, 1978).

A successful regeneration protocol depends on choosing the appropriate type of tissue to initiate the in vitro culture. In easily cultured nonlegumes such as carrot, almost any part of the plant taken at any developmental stage can serve as an explant source (Ammirato, 1983). In most legumes, however, the choice is quite limited. As a general rule, regenerable cultures are most easily obtained from immature tissue. Somatic embryogenesis from immature embryos or portions of embryos has been reported for white clover, peanut, and soybean (Maheshwaran and Williams, 1984; Sellars et al., 1990). Mature embryos have been used as explant sources for mung bean, pigeon pea, peanut, and soybean (Mathews and Rao, 1984; Mehta and Mohan Ram, 1980; McKently et al., 1990).

Various types of seedling tissue have been used as explant sources for a wide range of legume species. Seedling hypocotyls are perhaps the most widely utilized explant source for legumes, having been used for alfalfa, red clover, pea, pigeon pea, and several other species (Bingham et al., 1975; Phillips and Collins, 1979; Kumar et al., 1984; Oelck and Schneider, 1983). Epicotyls or cotyledons have been used for pea, red clover, alfalfa, and pigeon

pea (Malmberg, 1979; Phillips and Collins, 1980; Lupotto, 1983; Kumar et al., 1984).

Among mature tissue types, regeneration has been obtained from leaf explants in alfalfa and peanut (Oelck and Scheider, 1983; McKently et al., 1992). Mature petioles have served as explant sources in various clover species (Choo, 1988), and mature stems in birdsfoot trefoil and *Stylosanthes* (Swanson and Tomes, 1980; Meijer, 1981).

Several different basal medium formulations have been used for tissue culture of legume species. The majority of work has utilized Murashige and Skoog's (1962) MS medium (Malmberg, 1979; Kao and Michayluk, 1981; Hazra et al., 1989; McKently et al., 1990). Gamborg et al. (1968) developed the B5 medium for tissue culture of soybean, and this medium has proven useful for several other legume species, including red clover (Quesenberry et al., 1992) and birdsfoot trefoil (Swanson and Tomes, 1980). The B5 medium differs from MS primarily by its greatly reduced ammonium nitrogen level and a lower calcium concentration. Schenk and Hildebrandt's (1972) SH medium is similar to B5, but contains much higher levels of inositol. This medium has been used for *Stylosanthes* (Scowcroft and Adamson, 1976) and crimson clover (Horvath et al., 1979). Phillips and Collins' (1979) L2 medium was developed for red clover culture and has subsequently found application in the culture of peanut and soybean (Sellars et al., 1990), and alyceclover (Wofford et al., 1992a). This medium is somewhat higher in calcium than MS, B5, or SH, and also differs from these media in that it lacks nicotinic acid. Blaydes' (1966) medium contains less nitrate than the above media, and has been employed for soybean, alfalfa (Bingham et al., 1975), and pigeonpea (Kumar et al., 1984).

It is generally acknowledged that plant growth regulator levels are of critical importance in plant tissue culture. For angiosperms in general,

organogenesis can often be induced by "reversal transfer" of callus from a high auxin, low cytokinin to a low auxin, high cytokinin medium (Dodds and Roberts, 1985). Somatic embryos can often be induced on media containing high levels of auxin—particularly 2,4-D—sometimes in combination with low levels of cytokinin (Ammirato, 1983). Reviews of legume work (Allavena, 1983; Ammirato, 1983; Flick et al., 1983; Phillips and Collins, 1983) indicate that the above generalities are applicable. Earlier work favored use of kinetin to induce organogenesis, but more recently, BA (6-benzylaminopurine) , generally in the concentration range of 0.5 to 5.0 mg L^{-2}, has been widely used (Webb et al., 1987; Vieira et al., 1990; McKently et al., 1990). Somatic embryogenesis in legumes has been induced with 2,4-D concentrations ranging from 0.001 mg L^{-1} (Phillips and Collins, 1980) to 80 mg L^{-1} (Saunders et al., 1987).

In callus based regeneration systems, there is strong evidence that culture conditions for the initial establishment of callus can strongly influence the potential for subsequent organogenesis or embryogenesis (Saunders et al., 1985; Tisserat et al., 1978). This issue has received relatively little attention in legumes, and is generally approached empirically. Callus induction and regeneration can sometimes occur on the same medium (Santos et al., 1983; Bovo et al., 1986), but most work has employed a separate callus induction step (Phillips and Collins, 1979; Walker et al., 1979; Flick et al., 1983).

Little has been published specifically on tissue culture of desmodium. Angeloni et al (1988) produced multiple shoots from from shoot tip cultures of *D. incanum* on MS medium supplemented with 1.0 mg L^{-1} NAA (naphthalenacetic acid), 0.1 mg L^{-1} BA, and 1.0 mg L^{-1} GA (gibberellic acid). Shoot tip culture is regarded as meristem cloning rather than true regeneration, however. Wofford et al. (1992b) evaluated regeneration from seedling hypocotyl explants of six genotypes of *D. heterocarpon* and *D. ovalifolium*

under two protocols—an MS-based procedure intended to induce organogenesis and an L2-based procedure originally used by Collins and Phillips (1982) to induce embryogenesis in red clover. The MS procedure consisted of a callus induction step with 2.0 mg L^{-1} IAA (indole-3-acetic acid) and 1.0 mg L^{-1} kinetin, shoot induction with 0.1 mg L^{-1} IAA and 4.0 mg L^{-1} BA, and rooting on medium lacking plant growth regulators. The L2 protocol consisted of callus induction with 0.06 mg L^{-1} picloram (4-amino-3,5,6-trichloropicolinic acid) and 0.1 mg L^{-1} BA, embryo induction with 0.01 mg L^{-1} 2,4-D and 2.0 mg L^{-1} adenine, and embryo germination with 0.002 mg L^{-1} picloram and 0.2 mg L^{-1} BA. The MS protocol resulted in production of shoot meristems in five of six genotypes, but in all cases shoots failed to elongate. The L2 protocol yielded shoot meristems in two genotypes and whole plants were obtained in one genotype of *D. heterocarpon* ssp. *angustifolium*. The response on L2 superficially resembled organogenesis, but histological examination was not performed.

This chapter will evaluate a number of variations on the L2-based procedure utilized by Wofford et al. (1992b). Primary objectives were to determine if regeneration could be obtained in a wider range of desmodium genotypes and to optimize the response of previously identified regenerating genotypes. A bewildering range of culture variables can potentially influence regeneration response. The work presented here attempts to examine some of these variables in an orderly, stepwise manner in which the treatments in each experiment are based on the results of the previous experiment. The work begins with an examination of explant sources, proceeds to an investigation of various shoot bud induction treatments, and then examines several shoot elongation treatments. The chapter concludes with histological data presented with the objective of clarifying the mode of regeneration in this system.

Materials and Methods

Germplasm. Four genotypes were selected to represent a wide range of in vitro responses. In previous work (Wofford et al., 1992b) IRFL 6123 had been identified as a strong regenerator, CIAT 13083 as a possible regenerator, and UF 20 and UF 144 as nonregenerators. IRFL 6123 is classified as *D. heterocarpon* ssp. *angustifolium*, UF 20 represents *D. heterocarpon* ssp. *heterocarpon*, UF 144 represents *D. ovalifolium*, and CIAT 13083 possesses characteristics intermediate between *D. heterocarpon* ssp. *heterocarpon* and *D. ovalifolium*.

General protocol. Young, fully expanded leaves from greenhouse-grown plants were used to obtain leaf disc and petiole explants. Leaves and petioles were sterilized by immersion in 70% ethanol for 30 seconds followed by immersion in a 15% (v:v) Chlorox solution for one minute and several rinses with sterile deionized water. A stainless steel cork borer was used to cut 5 mm leaf discs, each containing a portion of midvein. Leaf discs were cultured with abaxial surface upward. Petioles were cut into sections approximately 10 mm in length.

For hypocotyl and cotyledon explants, seeds were scarified and sterilized by a 12-minute immersion in concentrated sulfuric acid followed by several rinses in sterile deionized water. Seeds were then placed in petri dishes containing SGL medium (Collins and Phillips, 1982) and incubated at 27° C. Hypocotyls and cotyledons were excised and placed onto callus induction medium after the hypocotyls reached a length of 7 to 10 mm. This occurred between 7 and 10 days after scarification. Hypocotyl explants were excised to a length of approximately 5 mm, with care taken to avoid radicle tissue and

cotyledon node tissue. Cotyledon explants consisted of the distal two-thirds of the cotyledon, placed abaxial surface upward.

All culture protocols used L2 basal medium (Phillips and Collins, 1980) solidified with 0.8 % (w:v) Phytagar (Gibco, Inc.; Island City, N.Y.). Growth regulators were coautoclaved with the basal medium for 20 minutes at 121° C. Cultures were maintained at 27° C with a 16/8 h light/dark cycle at an illumination of approximately 100 $\mu E\ m^{-2}\ sec^{-1}$. Callus induction and shoot bud induction was carried out in 60 mm disposable petri dishes with two or three explants per dish. For shoot elongation experiments, six calli were placed in each 100 mm petri dish.

All experiments utilized completely randomized designs. For number of shoots per explant, each explant comprised an observation. For percentage responding explants, each petri dish comprised one observation. Prior to statistical analysis, data on number of buds per explant were transformed to the square root of the quantity, observed value plus one half, in order to render variance independent of mean. Percentage data were transformed to arcsine of the square root of the observed percentage.

Evaluation of explant sources. Twenty-one leaf discs, petioles, hypocotyls, and cotyledons from each of genotypes UF 20, UF 144, IRFL 6123, and CIAT 13083 were cultured in a three-step protocol based on that described by Phillips and Collins (1980). Explants were placed on L2-based callus induction medium containing 0.06 mg L^{-1} picloram (4-amino-3,5,6-trichloropicolinic acid) and 0.1 mg L^{-1} BA (6-benzylaminopurine) for 28 days. Calli were then weighed and transferred to shoot induction medium containing 1.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} adenine, again for 28 days. Following the shoot induction treatment, calli with or without visible shoot buds were transferred to shoot elongation

medium with 0.002 mg L^{-1} picloram and 0.2 mg L^{-1} BA for another 28 days. Shoot bud production and callus appearance were evaluated regularly throughout the culture period.

Effect of 2,4-D level and kinetin level on shoot bud induction. Seedling hypocotyls from genotypes UF 20, UF 144, and IRFL 6123 were cultured for twenty eight days on callus induction medium as described above. Calli were then transferred to the following L2-based shoot bud induction media:

(1) 0.1 mg L^{-1} 2,4-D, 2.0 mg L^{-1} adenine
(2) 0.3 mg L^{-1} 2,4-D, 2.0 mg L^{-1} adenine
(3) 1.0 mg L^{-1} 2,4-D, 2.0 mg L^{-1} adenine
(4) 3.0 mg L^{-1} 2,4-D, 2.0 mg L^{-1} adenine
(5) 0.1 mg L^{-1} 2,4-D, 0.15 mg L^{-1} kinetin, 2.0 mg L^{-1} adenine
(6) 1.0 mg L^{-1} 2,4-D, 0.15 mg L^{-1} kinetin, 2.0 mg L^{-1} adenine

The 28-day shoot induction treatments were followed by a 28-day shoot elongation treatment as described for the previous experiment. Ten explants (two per dish) were used per genotype-treatment combination.

Induction of direct regeneration from hypocotyl explants without an intervening callus induction step. This experiment was carried out identically to the previous experiment with the exception that explants were placed directly onto the six shoot induction treatments rather than onto callus induction medium. Explants were rated after 28 days of shoot bud induction treatments and after 28 days on elongation medium.

Effect of duration of shoot bud induction treatment. Twenty-eight-day hypocotyl-derived calli from IRFL 6123 were transferred to shoot bud induction medium containing 2.0 mg L^{-1} adenine and either 1.0 or 0.1 mg L^{-1} 2,4-D for 0,

3, 7, 14, or 28 days. Following the induction treatment, calli were transferred to the shoot elongation medium described above. The 28-day induction treatment was followed by 28 days on elongation medium and the 14-, 7-, 3-, and 0-day induction treatments were followed by 42, 49, 53, or 56 days on elongation medium, respectively, to yield a total of 84 days in culture for each treatment. Calli were evaluated for bud formation at frequent intervals throughout the study. Twenty-one explants (three per dish) were used for each of the nine treatments.

Effect of BA and GA_3 (gibberellic acid) levels on shoot elongation. Hypocotyls from genotype IRFL 6123 were cultured on the callus growth medium described above for 28 days followed by 28 days on bud induction medium containing 2.0 mg L^{-1} adenine and 1.0 mg L^{-1} 2,4-D. Twenty-four calli, each containing at least one well-formed shoot bud or small bud cluster, were transferred to each of the following elongation media:

(1) 0.002 mg L^{-1} picloram, 0.2 mg L^{-1} BA, 0 mg L^{-1} GA_3
(2) 0.002 mg L^{-1} picloram, 0.2 mg L^{-1} BA, 0.2 mg L^{-1} GA_3
(3) 0.002 mg L^{-1} picloram, 0.6 mg L^{-1} BA, 0 mg L^{-1} GA_3
(4) 0.002 mg L^{-1} picloram, 0.6 mg L^{-1} BA, 0.2 mg L^{-1} GA_3
(5) 0.002 mg L^{-1} picloram, 2.0 mg L^{-1} BA, 0 mg L^{-1} GA_3
(6) 0.002 mg L^{-1} picloram, 2.0 mg L^{-1} BA, 0.2 mg L^{-1} GA_3

Shoot elongation was visually evaluated after 28 days of elongation treatment.

Effect of picloram/BA ratio on shoot bud elongation. This experiment was conducted in the same manner as the previous experiment, but with the following elongation treatments:

(1) 0.012 mg L^{-1} picloram, 0.2 mg L^{-1} BA
(2) 0.012 mg L^{-1} picloram, 0.6 mg L^{-1} BA
(3) 0.04 mg L^{-1} picloram, 0.2 mg L^{-1} BA
(4) 0.04 mg L^{-1} picloram, 0.6 mg L^{-1} BA

An additional elongation treatment of 0.002 mg L^{-1} picloram and 2.0 mg L^{-1} BA was included as a check.

Histological examination. After twenty-eight days of callus growth medium, calli of IRFL 6123 were transferred to shoot induction medium containing 1.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} adenine. Calli were removed for sectioning after 1, 2, and 3 weeks on induction medium. Specimens were embedded in Tissue-Tek O.T.C. Compound (Miles, Inc., Elkhart, IN) and sectioned on a CTF Microtome-Cryostat (International Equipment Co., Needham, MA). Sections eight microns in thickness were observed under the light microscope without staining.

Results and Discussion

Evaluation of explant sources. Wofford et al. (1992b) showed that seedling hypocotyls are satisfactory explant sources for in vitro regeneration in desmodium. The use of hypocotyls presents certain practical problems, however. Since the seedling must be dissected in order to place the hypocotyl

into culture, it is difficult or impractical to obtain both in vitro and in planta data from a single individual. Because an individual seedling possesses only a single hypocotyl, it is impossible to obtain replicated data on in vitro response unless quantities of genetically uniform seed are available. In the hope of eliminating these difficulties, an experiment was conducted to determine if regenerable callus could be produced using cotyledon, leaf disc, or petiole explants from genotypes IRFL 6123, CIAT 13083, or UF 20, with hypocotyl explants included as a check.

Results were discouraging. Cotyledons from IRFL 6123 and CIAT 13083 produced small amounts of necrotic callus while UF 20 cotyledons produced fairly abundant, deep green callus that showed no signs of regeneration. Leaf disc explants followed a similar pattern, with IRFL 6123 and CIAT 13083 producing meager quantities of nonregenerating callus primarily from major veins, and UF 20 producing larger quantities of nonregenerating callus. Petioles yielded somewhat greater callus mass than either cotyledons or leaf discs, but still failed to regenerate.

Hypocotyls yielded the greatest mass of callus from all genotypes. Ten of 21 hypocotyl-derived calli from IRFL 6123 produced shoot buds and elongated shoots were obtained from three of these. Shoot regeneration was observed from one UF 144 hypocotyl and one CIAT 13083 hypocotyl. In both cases, a single shoot bud appeared to form directly from explant tissue. This response could not be repeated in later experiments. It is possible that in both cases regeneration was the result of a small portion of meristematic tissue from the cotyledonary node being inadvertently included in the excised hypocotyl explants. Thus, shoots may have resulted from meristem cloning rather than true regeneration.

This work was rather limited in that the various explants were only tested under a single culture protocol. While it is possible that a different protocol might be more effective in inducing regenerable callus from leaf, petiole, or cotyledon explants, it is also possible that these explants would prove unresponsive to a wide range of protocols. In view of this uncertainty, further investigation of alternative explant sources was abandoned, and the remainder of this dissertation deals only with hypocotyl explants.

Effect of 2,4-D level and kinetin on shoot induction. The selection of treatments for this experiment were based on reports that regeneration in other legume species can be sensitive to 2,4-D concentration (Phillips and Collins, 1980; Saunders et al., 1987) and that low concentrations of kinetin in combination with 2,4-D can stimulate regeneration (Sellars et al., 1990).

None of the 2,4-D treatments, either with or without kinetin, resulted in shoot production in genotypes UF 20 or UF 144. Response of IRFL 6123 to shoot induction treatments is presented in Table 2-1. The presence of kinetin in the induction medium resulted in a slight browning of the callus and complete inhibition of shoot formation. In the treatments lacking kinetin, a clear response to 2,4-D concentration was observed. Analysis of variance (ANOVA) indicates that with respect to mean number of shoot buds per explant, 1.0 mg L^{-1} 2,4-D was superior to 0.1 mg L^{-1}, but not different from either 0.3 or 3.0 mg L^{-1}. Linear regression analysis of shoot buds per explant on 2,4-D level yields a significant quadratic relationship with a maximum between 1.0 and 3.0 mg L^{-1} (Figure 2-1). Thus, although ANOVA fails to indicate clear superiority of the 1.0 mg L^{-1} treatment, regression suggests that this treatment is close to the optimum 2,4-D level, and this is the concentration adopted for later work.

Consistency of regeneration response, as indicated by percent responding explants, is presented in the last column of Table 2-1. Although values in this

column span a wide range, no significant differences were found. Failure to detect statistically significant differences may be due to the low number of observations (10 per treatment) and the large number of nonresponding explants.

Direct regeneration. Direct regeneration from embryonic and seedling explants without an intervening callus phase has been demonstrated in several legume species (Ammirato., 1983). In an effort to achieve this in desmodium, the induction treatments applied to callus in the previous experiment were applied to newly excised seedling hypocotyls of three genotypes. Results were disappointing. Genotype UF 20 produced small quantities of callus on all treatments, but showed no sign of regeneration. No growth whatsoever was seen in UF 144. At 2,4-D concentrations of 0.1 and 0.3 mg L^{-1}, IRFL 6123 hypocotyls showed no growth. At the two higher 2,4-D concentrations IRFL 6123 produced roots from the radicle end of nearly every explant. When these were transferred to shoot elongation medium, tiny clusters of budlike structures less than 1 mm long formed at the end of the hypocotyl opposite the roots, but failed to develop further.

Effect of duration of shoot bud induction treatment. Since IRFL 6123 was the single responding genotype in the experiments described above, this experiment and the remainder of the chapter focus on optimizing protocols for this genotype. This experiment was designed not to enhance regeneration response, but rather to determine to what extent shoot bud induction period can be reduced without a corresponding reduction in regeneration.

Results presented in Table 2-2 show an increase in the mean number of shoot buds per explant through 14 days of induction and a leveling off thereafter. Despite the higher number of observations per treatment (21) than in the previous experiments, no significant differences were seen among the 3, 7,

14, and 28 day treatments with respect to percent responding explants. The zero day treatment yielded significantly fewer responding explants than all except the 7 day treatment. The zero day treatment is essentially a reversal transfer from high auxin, low cytokinin to low auxin, high cytokinin medium. Reversal transfer is a widely utilized method for inducing organogenesis. The rather poor response to this treatment relative to the 2,4-D treatments suggests that regeneration may be occurring via somatic embryogenesis rather than organogenesis. Histological evidence on this question will be presented later in this chapter.

The fact that a percentage of IRFL 6123 calli produce shoot buds under all treatments is evidence of this genotype's strong predisposition to regenerate. The failure of many of the explants to produce shoot buds under any of the treatments and the lack of significant differences among many of the treatments underscores the high level of variability often encountered in tissue culture work, and may also be an indication that we have not yet found—and may never find—the optimal regeneration protocol for this recalcitrant species.

Effect of BA and GA_3 levels on shoot elongation. Both shoot meristem formation and shoot elongation are difficult to induce in desmodium. In previous work (Wofford et al., 1992b) as well as in the experiments described above, a large percentage of shoot buds failed to develop into plants, either due to poor elongation or failure to root. Three levels of BA were evaluated in the first of two experiments intended to improve elongation response. Gibberellic acid has been reported to stimulate development and elongation of shoot meristems in cultured soybean and pigeonpea (Ghazi et al., 1986; Kumar et al., 1983). To determine if this compound might be useful for desmodium, each BA level was tested both with and without the addition of 0.2 mg L^{-1} GA_3.

Results are presented in Table 2-3. The two right-hand columns of this table represent two distinct types of elongation response. In the majority of cases shoot development ceased at the single leaf stage and at a length of one cm or less (Figure 2-2). These unifoliate shoots exhibited low vigor and could be rooted only with difficulty by transfer to basal medium supplemented with 1.0 mg L^{-1} indoleacetic acid (IAA). A smaller proportion of buds developed into multifoliate shoots (Figure 2-3). The more vigorous multifoliate shoots often spontaneously formed roots after two to three weeks in elongation medium, or could easily be induced to root upon transfer to basal medium lacking plant growth regulators.

Percentage of buds showing the unifoliate elongation response decreased with increasing BA level. In addition, a qualitative difference could be seen between the treatments with GA_3 and without. In the presence of GA_3, unifoliate shoots tended to bear abnormal, spatulate leaves (Figure 2-4). Because the degree of abnormality was variable and graded continuously into normal leaf development, abnormal and normal unifoliate elongation are not differentiated in Table 2-3.

Significantly more multifoliate shoots were produced on the medium containing 2.0 mg L^{-1} BA and lacking GA_3 than on the other treatments. The 21% multifoliate shoot production obtained with this treatment was nonetheless rather disappointing, and the following experiment was designed in an effort to improve on this figure.

Effect of picloram/BA ratio on shoot elongation. All of the treatments in the above experiment followed the precedent of Phillips and Collins (1980) and Wofford et al. (1992b) in utilizing the relatively low picloram concentration of 0.002 mg L^{-1}. Since neither increasing BA level nor addition of GA_3 produced dramatic improvement of elongation response, a second experiment was

performed to examine the effect of increasing picloram concentration and manipulating picloram/BA ratios. The most effective treatment from the previous experiment was included as a check. Results of this experiment are presented in Table 2-4. Just as in the previous experiment, unifoliate shoots substantially outnumbered the more vigorous multifoliate shoots. In contrast to the previous experiment, all of the leaves produced were relatively normal in morphology. No significant differences among treatments were observed for unifoliate shoot production, but the 0.012 mg L^{-1} picloram, 0.2 mg L^{-1} BA treatment yielded significantly more multifoliate shoots (38%) than the other treatments, including the best treatment from the previous experiment. It appears from these two experiments that when auxin level is insufficient, high cytokinin levels can serve a partial compensatory function in stimulating elongation. Superior elongation response can be obtained with a somewhat higher auxin level than that used in the original experiment in combination with fairly low level of cytokinin.

Histological examination of bud formation. As noted above, the method employed to induce regeneration in this study is consistent with an embryogenic pathway. The regeneration response appeared on a gross morphologic level to be organogenic. Histological examination revealed a mixture of these two regeneration mechanisms. At one and two weeks after transfer to induction medium, a low frequency of somatic embryos with clearly bipolar morphology and no vascular connections to the surrounding callus could be seen. These were mixed with a much higher frequency of clearly organogenic structures possessing a budlike morphology and definite vascular connections to surrounding callus. At three weeks and later stages embryos could no longer be found, while shoot buds at various stages of development were easily observed. Thus, it appears that regeneration in this system is predominantly via organogenesis.

Table 2-1. Response of genotype IRFL 6123 to 2,4-D and kinetin levels in the shoot bud induction medium.

2,4-D conc.	Kinetin conc.	Mean number of buds per explant †	Mean percent responding explants, %†
----------mg L^{-1}----------			
0.1	0	0.5 b	30 a
0.3	0	2.2 ab	70 a
1.0	0	3.3 a	80 a
3.0	0	1.6 ab	50 a
0.1	0.15	0 c	0 b
1.0	0.15	0 c	0 b

† Mean separation by Tukey's honestly significant difference (HSD α=0.05).

Table 2-2. Response of genotype IRFL 6123 to duration of shoot bud induction treatment.

Length of induction period, days	Mean number of buds per explant †		Mean percent responding explants, % †	
0	0.43	c	28	b
3	1.04	b	67	a
7	1.14	b	38	ab
14	2.52	a	57	a
28	2.50	a	61	a

† Mean separation by Tukey's HSD (α=0.05).

Table 2-3. Response of genotype IRFL 6123 to benzyladenine and gibberellic acid levels in the shoot elongation medium.

BA conc.	GA_3 conc.	Mean frequency responding explants † Unifoliate		Multifoliate	
----------mg L^{-1}----------		----------%----------			
0.2	0	50	a	0	a
0.2	0.2	54	a	0	a
0.6	0	33	a	4	a
0.6	0.2	38	a	0	a
2.0	0	13	b	21	b
2.0	0.2	13	b	0	a

† Mean separation by Tukey's HSD (α=0.05).

Table 2-4. Response of genotype IRFL 6123 to picloram and benzyladenine levels in the shoot elongation medium.

Picloram conc.	BA conc.	Mean frequency responding explants † Unifoliate		Multifoliate	
----------mg L^{-1}--------		----------%----------			
0.012	0.2	25	a	38	a
0.012	0.6	38	a	8	b
0.04	0.2	33	a	4	b
0.04	0.6	29	a	4	b
0.002	2.0	17	a	17	b

† Mean separation by Tukey's HSD (α=0.05).

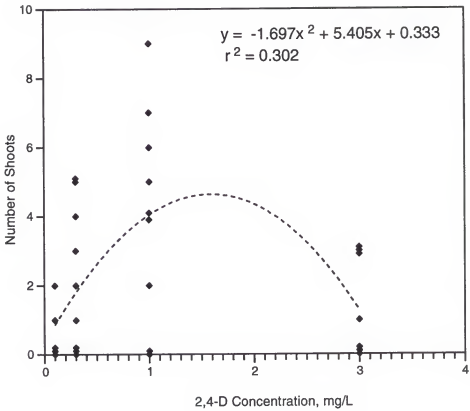

Figure 2-1. Response of genotype IRFL 6123 to 2,4-D concentration in shoot induction medium.

Figure 2-2. Unifoliate shoots produced by genotype IRFL 6123.

Figure 2-3. Multifoliate shoots produced by genotype IRFL 6123.

Figure 2-4. Spatulate shoots produced by genotype IRFL 6123 in the presence of gibberellic acid (GA_3).

CHAPTER 3
INHERITANCE OF IN VITRO REGENERATION AND ASSOCIATED CHARACTERS

The high genotype-specificity of in vitro regeneration in desmodium has been clearly demonstrated in the previous chapter. An understanding of the genetic control of regeneration is desirable for the efficient application of biotechnological methods to this crop. The mode of inheritance of regeneration determines whether it is feasible to transfer the trait to agronomically desirable nonregenerating lines and, if so, the appropriate method of accomplishing this.

The genetic basis of regeneration and associated in vitro traits has been studied in few higher plant species. In most studies regeneration was accomplished through organogenesis, and organogenesis is assumed in the current discussion unless otherwise stated. Regardless of whether organogenic or embryogenic response was examined, regeneration in most species was treated as a quantitatively inherited trait, although evidence supporting this assumption was often not presented. Several studies have been directed at partitioning genetic variance components and determining heritabilities for the regeneration trait in different crops. Buiatti et al. (1974) performed a diallel analysis, without reciprocals, of callus growth and shoot regeneration from flower petal explants in cauliflower. Additive gene effects were high for both traits, and narrow sense heritability estimates were 0.81 for callus growth and 0.09 for percent explants forming shoots. The low heritability for the latter trait resulted from extremely high epistatic and error effects. Based on North Carolina Design II matings among twenty four red clover genotypes, Keyes et al. (1980) determined that embryogenic regeneration frequency from

seedling hypocotyl-derived callus was subject to primarily additive genetic effects. Narrow sense heritabilities ranged from 0.25 to 0.54, depending on culture medium. Analysis of a complete diallel in tomato (Frankenberger et al., 1981) revealed that number of shoots produced per leaf disc explant was controlled predominantly by additive genetic effects, and the narrow sense heritability for this trait was estimated to be 0.98. A similar study in pigeonpea (Kumar et al., 1985) showed that callus growth was controlled largely by additive gene effects, while the number of shoots regenerated per cotyledon explant was subject to primarily nonadditive effects. Komatsuda et al. (1990) performed a detailed diallel analysis of callus proliferation and shoot regeneration from immature embryo explants in barley. Narrow sense heritabilities were approximately 0.7 for both traits. Significant dominant effects were detected for both traits, and significant epistatic effects were observed for shoot regeneration.

Evidence for qualitative inheritance of regeneration capacity has been reported in tomato, alfalfa and petunia. Koornneef et al. (1987) examined regeneration from leaf disc-derived callus in the progeny of a cross between tomato (*Lycopersicon esculentum*) and *L. peruvianum*. Segregation ratios in F_2, F_3, and backcross generations indicated that the trait was controlled by two dominant, complementary loci contributed by *L. peruvianum*.

Bingham et al. (1975) found that the frequency of regeneration from hypocotyl-derived callus in alfalfa could be increased from 12% to 67% in two cycles of recurrent phenotypic selection. Reisch and Bingham (1980) examined several F_1, F_2, and backcross populations produced from crosses between regenerating and nonregenerating clones of diploid alfalfa. Segregation ratios were consistent with a two gene model in which both genes were dominant and the presence of the dominant allele at either locus resulted in low frequency

regeneration, while the presence of both dominant alleles yielded a high regeneration frequency. Crosses between two different sets of nonregenerating clones yielded no regenerating progeny, suggesting that inheritance was not quantitative.

Wan et al. (1988) examined the genetics of regeneration from petiole explants in tetraploid alfalfa, employing a different culture protocol from that of Reisch and Bingham. Based on data from several F_1 and S_1 populations, it was concluded that regeneration was controlled by a two gene system distinct from that described by Reisch and Bingham. Duplicate recessive epistasis was postulated; that is, the presence of dominant alleles at both loci was necessary for any regeneration to occur. Variation in the frequency of regeneration among regenerating clones was attributed to either dosage effects or the presence of modifier genes.

Dulieu (1991) obtained rather tentative evidence of single gene control of regeneration from hypocotyl explants in petunia. Two backcross populations yielded approximately 1:3 ratios of "high regenerating" to combined "low regenerating" and "nonregenerating" plants, but the distinction between high and low level regeneration was somewhat arbitrary, and quantitative inheritance could not be ruled out.

All of the studies discussed above have dealt with regeneration from sporophytic (2n) tissue. The process of androgenesis, or embryogenesis from tissue derived from gametophytic (n) pollen mother cells, is markedly distinct from regeneration from sporophytic tissue. However, the two processes are sufficiently similar that work conducted on the genetics of androgenesis may provide insight into the genetics of regeneration from 2n tissue. Several papers have been published on the genetic control of androgenesis in cereals, and all

have treated both callus growth and regeneration frequency as quantitatively inherited traits. Diallel analysis of androgenesis in both wheat (Lazar et al., 1984) and triticale (Charmet and Bernard, 1984) revealed highly significant GCA, SCA, and reciprocal effects. In both cases, the GCA effect was predominant, and the wheat study reported a narrow sense heritability of 0.6 for plantlet formation. Earlier work involving a different set of wheat lines (Bullock et al., 1982) found no significant reciprocal effects, underscoring the fact that the results of any genetic study are highly specific to the germplasm under consideration. Two studies conducted on the inheritance of androgenic ability in barley yielded similarly disparate results. A study involving inbred lines and reciprocal F_1 hybrids (Foroughti-Wehr et al., 1982) found significant differences among reciprocal crosses, while a similar study (Dunwell et al., 1987) involving some of the same germplasm found no reciprocal differences. The latter work included F_2 and backcross generations and concluded that plantlet production from anther culture was highly complex genetically, with large epistatic effects. The work of Dunwell et al. (1987) also points out the importance of clearly defining the response variable. When response was defined as number of green plants produced per anther (the most common practice), the dominance effect was large and positive, with no significant additive effect detected. When response was expressed as percent responding anthers, however, the dominance effect was negative and a large, positive additive effect was seen. This study also reported that variation among different spikes on the same plant exceeded variation due to genetic factors. This suggests that physiological status of the anther can be a major determinant of capacity for androgenesis. A diallel analysis in rice (Quimio and Zapata, 1990) found that callus production and number of regenerated plants per anther were both influenced primarily by additive genetic effects, with no significant reciprocal effects. Variance (V_r) and

covariance (W_r) analysis indicated that regeneration was largely controlled by partially recessive genes.

The limited number of studies that have addressed phenotypic or genetic correlations between in vitro regeneration and various in vitro and in planta characters have yielded both expected and unexpected results. Oelck and Scheider (1983) found that in *Melilotus officinalis*, *Trifolium pratense*, and *T. resupinatum*, the ability to form shoots from callus was correlated with the production of side shoots from in vitro shoot tip cultures. Kern et al. (1986) observed a similar correlation between capacity for somatic embryogenesis and in vitro axillary shoot development in soybean. These correlations may reflect a generalized tendency among responding genotypes to form shoots, or may simply indicate a tolerance to the specific culture conditions employed in the study. It has been observed in alfalfa (Bingham et al., 1975; Brown and Atanassov, 1985) and alyceclover (Wofford et al., 1992a) that regenerating genotypes often have a creeping growth habit and readily produce adventitious shoots. Thus it appears that in at least some cases the ability to regenerate in vitro reflects a general proclivity for shoot production both in vitro and in planta. Oelck and Scheider (1983) suggested that a general tendency to produce adventitious shoots may be a useful indicator in preliminary screenings of germplasm for regenerating lines.

Perhaps surprisingly, there appears to be little correlation between callus growth rate and regeneration frequency (Baroncelli et al., 1974; Kumar et al., 1985; Lazar et al., 1984). However, shoot regeneration from callus can be restricted in genotypes with very poor callus growth (Bingham et al., 1975). It is well established that callus appearance frequently bears a strong relationship to regeneration potential (Bingham et al., 1975; Ketchum et al., 1987; Delieu

1991), but due to the difficulty of quantifying callus appearance the relationship has not been subjected to statistical analysis.

The primary objective of the work described in this chapter was to determine the mode of genetic control of in vitro regeneration in desmodium. A secondary objective was investigation of the mode of inheritance of callus growth and examination of the relationship between callus growth and regeneration.

Materials and Methods

Production of hybrids. Two regenerating and three nonregenerating lines were selected for crossing. The regenerators, IRFL 6123 and IRFL 6128 are classified as *D. heterocarpon* ssp. *angustifolium*, and are morphologically distinguishable from the nonregenerating genotypes by their lanceolate, coriacious leaves, upright growth habit, elongated racemes, and glabrous seed pods. Lines 6123 and 6128 are very similar except for a distinct leaf mark on 6123 that is absent on 6128. All of the selected nonregenerators—UF 20, UF 144, and CIAT 13083—have ovate or obovate, noncoriacious leaves, spreading growth habits, compact racemes, and pubescent pods. Genotype UF 20 ('Florida' carpon) represents *D. heterocarpon* ssp. *heterocarpon* and is distinguished by its thin, slightly pubescent leaves and prominent leaf marks. Genotype UF 144 is classified as *D. ovalifolium*, and possesses thicker, glabrous leaves. Genotype CIAT 13083 is intermediate between *D. heterocarpon* and *D. ovalifolium*.

Parent plants were moved from the field to the greenhouse in April, 1989. To induce flowering, a ten hour day length was simulated by covering the plants with a tarpaulin from approximately 7:00 P.M. until 9:00 A.M. Pollinations were

done in June and July. Donor pollen was obtained by tripping flowers using a toothpick with a small piece of fine sandpaper glued to one end in such a way that the anthers and stigma would strike the sandpaper and pollen would adhere. Pollen was then transferred to recipient flowers by tripping in a similar manner. Recipient flowers were not emasculated because the flowers were extremely sensitive to handling, and emasculation tended to result in flower abscission. Pollinations were carried out at a time of day when the flowers had fully opened, but self-tripping had not yet occurred. The specific time varied according to night temperature and daytime cloud cover, but was generally between 9:00 A.M. and noon. At least one hundred flowers for each possible combination of regenerator and nonregenerator parent, including reciprocals, were pollinated in this manner.

Seeds were harvested in August,1989 and germinated and planted in the greenhouse the following winter. Hybrids were clearly identifiable based on the morphological characteristics described above. Flowering of hybrids was induced in the summer of 1990. Plants were allowed to self-pollinate, but flowers were hand tripped to increase seed set.

Evaluation of callus growth and regeneration in the parental, F_2 and F_3 generations. The F_2 seed harvested from the original hybrids was scarified and germinated on SGL medium (Collins and Phillips, 1982) as described in Chapter 2. Hypocotyls were excised and placed onto callus induction medium and epicotyls were returned to SGL medium for rooting. Rooted epicotyls were transferred to potting soil after ten days and grown to maturity in the greenhouse where they were allowed to self-pollinate to produce the F_3 generation.

Hypocotyls from both F_2 and F_3 populations were cultured according to the optimal protocol established in Chapter 2. Initial callus production was on L2 medium supplemented with 0.06 mg L^{-1} picloram and 0.2 mg L^{-1} BA for

28 days. Shoot buds were induced on L2 with 1.0 mg L^{-1} 2,4-D and 2.0 mg L^{-1} adenine for 14 days, and shoot elongation was induced on L2 with 0.012 mg L^{-1} picloram and 0.2 mg L^{-1} BA for 28 days. Shoots were rooted on L2 lacking plant growth regulators. Hypocotyls from parental lines were cultured simultaneously with the F_2 and F_3 populations to serve as checks.

Callus fresh weights were measured at the time of transfer from callus medium to bud induction medium. Each callus was visually scored for shoot bud formation and elongation at the end of both the bud induction and bud elongation steps. The scoring system and statistical transformations used will be discussed in the results.

Statistical analysis. General statistical analyses were conducted using JMP Version 3.0, the SAS Institute general statistics product for Macintosh computers (SAS Institute, 1994). Means and variances of truncated data sets were estimated using UNCENSOR Version 3.0 (Newman and Dixon, 1989).

Results and Discussion

Results of crossing efforts were disappointing. The approximately 2200 flowers that were cross-pollinated produced only 590 seeds. Poor seedset was likely due in part to inadequate greenhouse ventilation, which resulted in occasional daytime temperatures sometimes exceeding 38° C. Hybrid yield was further reduced by the low ratio of hybrids to selfs in the seeds that were produced. Approximately 550 of the 590 seeds germinated, but only eight of the resulting plants were identified as hybrids. Hybrids could be readily identified by leaf shape and growth habit, both of which were intermediate between the parental types. Two hybrid plants were progeny of the cross IRFL 6123 x CIAT 13083 (hereafter referred to as cross 501), three were from cross

IRFL 6123 x UF 144 (cross 507), and three were from CIAT 13083 x IRFL 6128 (cross 510). Since genotypes 6123 and 6128 are nearly identical, both in gross morphology and in vitro performance, crosses 501 and 510 are essentially reciprocals.

Hybrids were selfed through the F_3 generation. Table 3-1 summarizes numbers of individuals and number of families from which callus growth and regeneration data were collected. In the case of cross 510, data collection was terminated at the F_2 generation. Severe morphological abnormalities appeared in the F_1 of this cross, and persisted through F_3. The three F_1 seedlings appeared normal through approximately 6 weeks of age, after which new growth showed severely stunted leaves and internodes. Stunting was evident from the early seedling stage in many F_2 and F_3 individuals, and appeared at late seedling stages in all individuals. Stunting was accompanied by extremely poor flower production and seedset. The F_2 of cross 510 also exhibited a depressed callus growth rate relative to the two other crosses and to the parental lines , with a high incidence of callus necrosis and death. Since cross 510 is essentially the reciprocal of cross 501, the abnormalities may be the result of interactions between the cytoplasm of CIAT 13083 and nuclear genes of IRFL 6127. (Evidence of maternal effects in the other crosses will be discussed later in this chapter.) Whatever the cause, the observed abnormalities have the potential effect of masking expression of the in vitro characters of interest, as well as exerting confounding selective pressures in the F_2 and F_3 generations. Therefore, genetic analysis was not attempted on cross 510, and the remainder of this chapter deals exclusively with crosses 501 and 507.

Scaling of the callus growth trait. The logical first step in genetic analysis of any trait is to identify an appropriate numerical scale; that is, to determine if

mathematical scale transformation is necessary. This task can be approached in at least two general ways. A purely statistical approach, described in any number of statistics texts (Little and Hills, 1978; Zar, 1984), seeks a scale that yields a data structure satisfying the general assumptions of parametric statistics—in this case, normally distributed error terms with error variances independent of means. A more genetically oriented (and somewhat more complex) approach is not directly concerned with statistical assumptions, but stresses instead whether a scale will facilitate partitioning of genetic variation into underlying genetic causes, or components; e.g., additive, dominant, and epistatic gene action (Mather and Jinks, 1971). Implicit in this second approach is the statistical assumption that factor levels act in an additive fashion. Both approaches will be presented below.

Three scales were examined in the present study. The simplest is the untransformed linear scale, which in this case is callus weight in grams. This scale possesses the virtue of allowing various statistics and parameters to be reported in grams, permitting easy interpretation. A logarithmic scale was also investigated. While this scale results in somewhat non-intuitive statistical outputs, the scale is theoretically more applicable to analysis of growth variables than is a linear scale. The logarithmic scale asks the question, how many times has the cell mass doubled, while the linear scale asks how many mass units have been added to the starting mass. The logarithmic transformation is frequently used to stabilize variances in cases where standard deviation is proportional to mean in the untransformed scale. The third scale tested was the square root transformation. This scale is intermediate between the linear and logarithmic scales in terms of skewing of distributions, and is useful when variance is proportional to mean.

To examine the effect of the three scales on normality, the scales were applied to populations of each of the three parental lines and to F_2 and F_3 populations. The parental populations are theoretically normally distributed, while the F_2 and F_3 populations are expected to be normally distributed if little or no unidirectional dominant or epistatic gene action is present. The later generation populations are included here because, as a result of a temporarily limited seed supply, the size of the tested parental populations was quite small ($20 \leq n \leq 36$). Univariate statistics for the various populations are presented in Table 3-2. The linear scale tends to produce rightward (positive) skewing, while the log transformed populations are moderately left-skewed (negative), and the square root transformed populations are slightly left-skewed. The Shapiro-Wilk W statistic is used to test for normality in populations as small as ten observations; a low probability associated with a calculated value of W indicates a significant deviation from normality (SAS Institute, 1989; Gilbert, 1987). None of the W values for the parental populations indicated deviation from normality.

Results for F_2 and F_3 populations are similar to those for the parental populations, but are somewhat clearer. The linear scaling results in gross leftward skewing and yields highly significant W statistics. This may be attributable to genetic effects if there is a strong preponderance of negatively acting dominant genes in these populations. Strong, unidirectional epistatic effects, which could result in a relatively small proportion of the populations possessing the necessary combination of alleles for rapid callus growth, is another possible genetic cause for the observed skewing. However, the fact that the leftward skewing occurs in all populations can be interpreted to indicate that the linear scale is inadequate and that with proper scaling the F_2 and F_3

will in fact be approximately normally distributed. Further evidence regarding this hypothesis will be presented later in this chapter.

Skewness and W statistics for the F_2 and F_3 populations fail to clearly indicate whether the logarithmic or the square root transformation yields a more normal distribution. As in the case of the parental populations, the log transformed populations are skewed to the right, while the square root transformed populations are skewed to the left. In the case of F_3 populations, the magnitude of skewing is substantially less for the logarithmic than for the square root scales. The only non-significant W statistic was obtained from the square root transformed cross 507 F_2 population.

If a variable is properly scaled, populations with high means should, in general, have variances no greater than those of populations with lower means. This can be tested by regressing F_3 family means versus variances, as presented in Figure 3-1. The linear scale exhibits a strong, positive relationship between mean and variance. Logarithmic transformation results in a weak, negative relationship, and the square root transformation produces a weak, positive relationship. It is feasible that the expectation of independence of means and variances can be confounded by genetic effects, since different F_3 families may possess differing amounts of genetic variance. For example, in the not unlikely situation that a large, positive dominance effect exists, families with high means will be those whose F_2 parents had high levels of heterozygosity for genes controlling callus growth. These families will also exhibit elevated variances due to the presence of segregating genes. This genetic effect can only be large, however, if total genetic variance is much greater than environmental variance. It will be shown later in this chapter that this is not the case. Therefore, the strong relationship between means and variances for F_3 families provides further evidence of the inadequacy of the linear scale.

Because only three parental populations were examined, meaningful regression of mean versus variance for parental populations is not possible. However, simple examination of parental means and variances (Table 3-2) can shed further light on scale effects. If variance is independent of mean, the variances of the three parental populations should be similar in spite of the differences in means. The effects are similar to those seen in the F_3 populations, but the square root transformation stands out in that it yields a smaller relative range in variances (maximum variance 48% greater than minimum) than either the linear (177%) or logarithmic (78%) scales.

In summary, a general statistical examination of the callus growth trait reveals the clear inadequacies of linear scaling. Logarithmic and square root scales are similar in their conformity to the statistical requirements of normality and independent variance.

Joint scaling test for callus growth. A more genetically oriented approach to scaling was developed by Cavalli (1952) and elaborated upon by Mather and Jinks (1971). Known as the joint scaling test, in its simplest form this technique involves estimating the effects of additive and dominant gene action on generation means. This is done by establishing a series of simultaneous equations, one for each generation for which data are available. The left side of each equation is the observed generation mean. The right side consists of three terms, each of which is composed of an unknown and an associated coefficient. The unknowns are model parameters: the grand mean (essentially the genetic-neutral component of the various generation means, designated as m), additive genetic effect (designated [d]), and dominant genetic effect (designated [h]). Coefficients correspond to the theoretical causal contributions made by these parameters to each generation mean. Since there are three unknowns, data from at least four generations (each parent represents one

generation) are needed to obtain a non-singular solution and a measurement of statistical significance of the solution. Each equation is weighted in proportion to the level of certainty associated with each generation mean, which is equivalent to the reciprocal of the variance of the mean. As originally described, a solution is obtained through cumbersome matrix manipulations. This is computationally identical to performing weighted least-squares regression using the observed means as the dependent variable and the model coefficients as independent variables (Rowe and Alexander, 1980). The partial regression coefficients obtained in this way are equivalent to the unknowns in the above-described equations. The adequacy of solutions obtained by this method is assessed in two ways. First, each predicted model parameter has an associated F test for the hypothesis that the parameter differs from zero. Secondly, the whole model can be tested by examining the error sum of squares (SSE). In this particular form of analysis, SSE exhibits a χ^2 distribution, and the reported SSE can be compared to tabular χ^2 values to determine goodness of fit (Rowe and Alexander, 1980).

Six joint scaling tests were conducted in order to test each of the three scales using data from each cross. Model coefficients used to construct the tests are given in Table 3-3. Observed means and weights used in the tests are are based on the means and variances presented in Table 3-2. Predicted values of m, [d], and [h], and the results of goodness of fit testing are given in Table 3-4. Adequate goodness of fit is obtained only for the linear and square root scales and only for cross 501. In both cases, a large, negative dominance effect is indicated.

The joint scaling test suffers from limitations that necessitate cautious interpretation of results. One serious limitation is that the test assumes that one parent possesses most of the positively acting alleles and the other possesses

the majority of negatively acting alleles, and that dominance acts primarily in a single direction. Judging from the large difference in callus growth rates among the parents in the current work, the first assumption is probably at least partially valid. The validity of the second assumption is difficult to assess. A second problem with the joint scaling test is that two separate hypotheses are simultaneously tested: that the scale is adequate, and that the genetics of the trait in question can be adequately described by an additive-dominant genetic model. When an adequate goodness of fit is not obtained, the joint scaling test cannot by itself distinguish which of these hypotheses is incorrect. It is possible to partially solve this problem by adapting the test to more complex genetic models involving epistatic and perhaps other types of genetic effects. Unfortunately, more complex models require data from more generations and are dependant on an increasing number of rather dubious assumptions about gene interactions (Mather and Jinks, 1971). As a result of these limitations, spurious positive results are quite possible, particularly when the test involves only a single degree of freedom. Considering the statistical evidence for the inadequacy of the linear scale, it is likely that the good fit obtained when this scale was applied to cross 501 is in fact spurious.

Examination of the data presented in Table 3-2 suggests a factor that may confound the joint scaling test in the present case. In both crosses, and for all scales, the F_2 and F_3 means are significantly lower than the mid-parent mean, and are fairly close to one another. In the case of cross 501, the F_3 mean is higher than the F_2 mean, while in cross 507 the F_3 mean is lower. If a simple additive-dominant model is assumed, the deviation of the F_2 and F_3 from the mid-parent mean suggests a large, negative dominance effect, just as indicated by the joint scaling test. However, the relationship between F_2 and F_3 means suggests a small dominance effect—a negative effect for cross 501, and a

positive effect for cross 507. The discrepancy between the dominance effect predicted by the relationship of the progeny means to the mid-parent mean and that predicted by the relationship between F_2 and F_3 means accounts for the generally poor joint scaling test results. These observations suggest that some factor not considered in the model may be acting to depress both F_2 and F_3 means relative to parental means. This factor could be a maternal or cytoplasmic effect—nuclear genes from parents 144 and 13083 may interact in a negative manner with cytoplasmic genes from 6123 (the female parent in both crosses). The depressed callus growth observed in cross 510 is consistent with this explanation. An alternative cause would be negative epistatic interactions between nuclear genes originating from the different parents. If either explanation is correct, then the results of the additive-dominant joint scaling tests are invalid. Unfortunately, there are insufficient generations available to allow construction of an additive-dominant-maternal or additive-dominant-epistatic joint scaling test.

Variance partitioning for the callus growth trait. Variance partitioning methods are not subject to all of the limitations described for the joint scaling test. Specifically, variance partitioning is not affected by maternal factors that affect means, by distribution of positive-acting alleles between parents, nor by dominance acting in different directions at different loci. A sophisticated method of deriving genetic variance parameters from observed variances and covariances in segregating generations derived from crosses of inbred lines is presented by Mather and Jinks (1971). The technique is similar in many ways to the joint scaling test, but observed variances and theoretical causal variance components are utilized to construct the model. As with the joint scaling test, fairly complex genetic models are possible, but, due to data limitations, the present discussion will be limited to a simple additive-dominant model. In the

present case, five observational variances or covariances are available: pooled parental variance (E_1), F_2 variance (V_{1F2}), among-family F_3 variance (V_{1F3}), within-family F_3 variance (V_{2F3}), and F_2-F_3 covariance (W_{1F23}). Among- and within-family F_3 variances are obtained by analysis of variance using F_2 parent as the grouping variable, followed by partitioning of variances based on expected mean squares (Appendix A). The F_2-F_3 covariance is the covariance between F_2 parents and F_3 family means.

The causal variance components described by Mather and Jinks are somewhat complex. In addition to additive and dominant genetic components (designated D and H, respectively), two types of environmental variance and a so-called sampling variance are described. The first environmental variance (E_W) is simply the error variance among individuals. The second (E_B) is the error among plots or rearing environments and is applicable only to V_{1F3} in the present study. Since explants from the different F_3 families were randomly distributed among petri dishes, which were randomly distributed within a single incubator, there is no intrinsic "plot" error. Instead, E_B is equivalent to E_W divided by the harmonic mean of the number of individuals per F_3 family (Mather and Jinks; 1971). Since E_B is a function of E_W, it can be combined into E_W, thereby eliminating a column from the model and gaining a degree of freedom. The sampling variance applies only to among-group variances. It is similar in concept to E_B, but is a function of within-group genetic variances rather than error variances. Therefore, in the case of V_{1F3} the sampling variance is equivalent to V_{2F3} divided by the harmonic mean of the number of individuals per F_3 family. Sampling variance can be algebraically included into the model row for V_{1F3} and does not have an associated model column. Derivation of model matrices is described in detail in Appendix B.

The method of Mather and Jinks employs an iterative approach for determining model weights. Weights for each of the matrix rows are initially established as the reciprocals of the theoretical variances of each observed variance or covariance. The variance of a variance is equal to $2(V^2)/df$, where V is the observed variance and df is the number of degrees of freedom on which the observed variance is based. The variance of a covariance is equal to $(W^2 + V_1V_2)/df$, where W is the observed covariance, V_1 and V_2 are the variances of the populations from which the covariance was obtained, and df is the degrees of freedom upon which the covariance is based. The model is then solved, and predicted (Mather and Jinks use the term expected) observational variances are obtained. These are used to derive new weigh estimates, and the model is solved again. This is continued until no improvement in model fit is obtained.

Coefficients used in variance partitioning for the callus growth trait are presented in Table 3-5. Observational variance components and initial weights are presented in Table 3-6. Results of the first iterations are given in Table 3-7. In all cases, second and third iterations failed to improve model fits. (Results of later iterations are presented in Appendix C.) The linear and logarithmic scales failed to produce significant whole-model F tests for either cross. The square root scale produced significant whole-model tests for both crosses. For cross 501, estimated additive genetic variance was large, and significantly different from zero (assuming a rather generous α of 0.1), while dominant variance was smaller and not significantly different from zero. Cross 507 yielded no significant genetic variance estimates.

The analysis presented thus far gives a rather unclear and somewhat contradictory picture of the genetic mechanism controlling the callus growth trait in the two crosses. Both the joint scaling and variance partitioning approaches

indicate that cross 507 cannot be adequately described by an additive-dominant genetic model. Based on the joint scaling test, the genetics of cross 501 may be characterized by a large, negative dominance effect and a moderate additive effect. An alternate interpretation is that large, negative maternal or epistatic effects exist in both crosses. In contrast, variance partitioning indicates a large additive genetic variance and a much smaller dominant variance in cross 501.

A general shortcoming of the types of analysis presented to this point is the large number of simplifying genetic assumptions required. The results derived from variance partitioning may be more reliable than those of the joint scaling test, due to the fewer genetic assumptions required by this method. Many assumptions, such as absence of linkage and equal magnitude of effect from each contributing locus, are common to both techniques. The generally poor model fits obtained by both methods may be due to failure of the experimental material to conform to these assumptions, or to the presence of significant nonadditive, nondomininant genetic effects. It is likely that experimental error also contributes to poor model fit.

Parent-offspring regression is a simpler, more empirical method of analysis, relatively free of genetic assumptions. Regressions of F_3 family means on F_2 parents are presented in Figure 3-2. The slopes (regresion coefficients) represent, by definition, heritabilities. It should be noted, however, that in the case of selfing, parent-offspring regression is in part a function of dominant and epistatic genetic effects, but these effects play a much smaller role in the regression than does additive genetic variance. Regression coefficients are remarkably high, regardless of cross or scale, ranging from 0.524 to 0.769. The practical implication of this is that substantial progress

should be possible in selecting for callus growth among segregating generations of this material.

The nature of the regeneration variable. The callus growth trait was amenable to quantitative analysis due to the fact that this trait could be expressed in terms of a continuous, metric variable for each individual plant. This was not the case for the regeneration trait. In an ideal study of the genetics of regeneration, each plant can be evaluated in terms of a metric variable such as percent responding explants or number of shoots per explant. Because hypocotyl explants had to be used in the present study, only one explant existed per plant, so the data could not be expressed as percent responding explants. Expression of regeneration as number of shoots per explant was impossible because the majority of explants produced no buds or shoots. Instead, the trait was expressed on a visual rating scale of one through seven. A rating of one represented no evidence of regeneration. Two represented a slight indication of regeneration in the form of localized deep green coloration. Three represented formation of a single, well-defined but nonelongated bud. Four indicated multiple bud formation, usually with some elongation. A rating of five indicated one or two well elongated shoots. Six indicated between three and five elongated shoots, and a rating of seven indicated more than five elongated shoots.

A significant number of individuals in all populations showed no sign of regeneration and received scores of one. All individuals in the nonregenerating parent populations (genotypes 144 and 13083) received scores of one. This type of data structure can be described as "threshold" or "censored" data. Falconer (1981) discussed threshold traits at length. He assumed that there exists an underlying, continuous scale of proclivity (or as Falconer describes it, "liability") towards a certain condition—in the case of the present work, tendency

to regenerate—that can only be detected when the level of proclivity rises above a given threshold.

Distributions of this type cannot be rendered normal by any mathematical transformation, since we lack any information about differences among those individuals that fall below the observable threshold, and no transformation can restore this missing information. For example, no matter what transformation is used, the two nonregenerating parental populations will always have equal means, and variances of zero. Fortunately, useful analysis can still be performed on this type of data, provided it can be assumed that the populations under consideration are normally distributed.

In Falconer's treatment, threshold traits can be manifested at either two or three discrete levels, or classes. This can be done with the present data by collapsing the rating scale into two (nonregeneration versus regeneration), or three (nonregeneration versus partial regeneration versus full regeneration) classes. While little useful analysis can be done when only two classes are present, meaningful analysis is possible in situations with three classes, contingent on the assumption that the underlying variable is normally distributed. For any population, if the percentage of individuals falling into each of three threshold classes is known, then the mean and variance of the population, expressed in threshold units, can be deduced. The principle is illustrated in Figure 3-3, adapted from Falconer (1981), but based on actual F_3 data. The classes are separated by the two vertical lines on each graph, nonregenerators lying to the left of both lines, partial regenerators lying between the lines, and full regenerators lying to the right. While both populations contain 68% full regenerators, population (a) contains more partial regenerators and fewer nonregenerators than population (b). As a result,

population (a) has a lower standard deviation and lower mean than population (b).

An attractive advantage of analyzing data based on three ordinal classes with two thresholds is that scaling problems are avoided. This is because only one real unit exists on the x axis—the unit separating the two thresholds. Values either to the left or to the right of both thresholds can never be experimentally measured, and are simply extrapolations of the central threshold unit. A serious disadvantage of the three class approach is that populations that do not contain at least one observation in each of the three classes cannot be analyzed. Many of the F_3 families in the present study contain no fully regenerating individuals, and therefore cannot be analyzed using this approach.

While the methods described by Falconer for analysis of threshold traits cannot be directly applied to data with more than three classes of observations, it is possible to analyze data with an unlimited number of classes by applying techniques developed for censored data sets. Censored data are similar to threshold data in that both data types are subject to a threshold below which no information is known (Schneider, 1986). The difference is that in censored data there is only one threshold, and data above this threshold are of a continuous, or at least semicontinuous nature. In both cases, data lying above the detection threshold are used to make inferences about data below the threshold; therefore, assumptions of normality are necessary in both cases. By treating the seven-category regeneration rating scale as a quasi-continuous variable, the data collected in the present study are amenable to analysis as censored data.

Several statistical techniques have been developed for "uncensoring" censored data sets, and, as in many fields of statistics, the relative strengths and

weaknesses of each are subject to considerable debate among statisticians. The most intuitively appealing method is termed regression order statistics. This method is graphically illustrated in Figure 3-4. Figure 3-4 (a) is a normal probability plot of a hypothetical, approximately normally distributed population. The Y-axis is observed value of the variable of interest, and the X-axis is normal quantile score (a transformed Z-score scale). The points represent the individual observations, and the line represents the simple linear regression line obtained from the individual data points. The slope and intercept of this line can be used to approximate the variance and mean of the population. Figure 3-4 (b) is a similar plot of the same population with a censoring threshold applied such that approximately 30% of the observations yield no observable response. These observations, of course, all have the same values for both axes, and therefore all lie atop one another at the lowest left data point on the graph. The result is a truncated plot. The regression line is derived only from those observations lying above the censoring threshold, but is similar to the line obtained from the uncensored population, and would yield variance and mean estimates close to the actual parameters of the original population. The regression order statistics method is a numeric analog of Figure 3-4 (b). That is, it performs a normal quantile regression on a truncated population in order to approximate the mean and variance of the intact population.

The accuracy of population parameters estimated by uncensoring through regression order analysis is limited by the number of observations lying above the censoring threshold. However, reasonably accurate results can be obtained even when the majority of observations are below the threshold, as long as several observations lie above the threshold. Regression order analysis was used to estimate means and variances of all populations in the current study that had at least four individuals with regeneration ratings higher

than one. Although this is a less than ideal approach, there appears to be no acceptable alternative. The option of simply analyzing the raw regeneration scores—either transformed or otherwise—is undesirable because this entails accepting the clearly incorrect assumption that all individuals receiving regeneration scores of one have the same proclivity for regeneration. The contrasting options, then, are to ignore the left-hand tails of many of the population distributions, or to use an acknowledgedly problematic method of reconstructing these tails. Means and variances determined by uncensoring, as well as means and variances of the raw data are presented in Table 3-8. In general, means derived from regression order analysis are lower than those obtained from the raw data, and variances are higher. This is not unexpected, since truncation of the low end of a data set—as occurs in the raw data—will artificially raise the mean and lower the variance. For the raw data, a significant (α=0.05), positive relationship exists between F_3 family variance and family mean, due at least in part to the fact that the lower the mean, the more severely truncated the distribution. This relationship is absent from the uncensored data because the truncated lower ends of the distributions have been reconstructed. The unusually high variances observed for some F_3 families may be due to segregation of major genes within these families, resulting in a somewhat bimodal distribution and exaggerated variance. This effect is most pronounced in the uncensored data. Unfortunately, population sizes for the F_3 families are too small to clearly distinguish between spurious bimodality and bimodality resulting from genetic causes. Bimodality was not observed in the F_2 populations, nor in the complete F_3 populations.

The accuracy of the variance estimates presented in Table 3-8 can be compromised by non-normality resulting from inadequate scaling. Major scaling problems can be detected by examining the distribution of a population

containing no genetic variance (since genetic factors can also cause deviations from normality) and having a mean significantly higher than the censoring threshold. The regenerating parental population (genotype 6123) is such a population, and a histogram of this population is presented in Figure 3-5. The distribution is not seriously skewed, nor is it bimodal, suggesting that there is little scale-induced non-normality.

Genetic analysis of the regeneration trait. Due to the difficulties presented above, the types of analysis that can be performed on the regeneration trait are severely limited. The joint scaling test cannot be used because this test requires an estimate of the mean for each parent. Neither of the nonregenerating parent populations (genotypes 144 and 13083) gave any evidence of regeneration, so meaningful estimates of mean regeneration score could not be obtained for these parents. This reiterates the case for analyzing the uncensored rather than raw regeneration data. All calli for each nonregenerating parental genotype received regeneration scores of one, so based on the raw data the two genotypes have equal mean regeneration scores. There is no reason to believe that both of the nonregenerating parents have the same genetic proclivity for regeneration. The scores merely indicate that neither genotype has enough proclivity to produce any visible evidence of regeneration under the culture protocol used.

The iterative variance partitioning method used for the callus growth trait could not be applied to the regeneration trait. This method requires good estimates of the uncertainty associated with variance estimates and it was felt that these were lacking for the regeneration trait. Mather and Jinks (1971) described a simpler, nonweighted, noniterative variance partitioning method. Preliminary efforts were made to apply this method to the regeneration trait. Both raw and uncensored data were examined in this way, despite the

truncation-related biases in the raw data. Preliminary variance partitioning yielded no significant (or even nearly significant) estimates for any parameters. In the case of cross 501, this appeared to be due to an excessively high within-family variance in the F_3 and extremely low among-family F_3 variance and F_2-F_3 covariance. For cross 507, the primary confounding factor appeared to be an overly high F_2 variance. In both cases these factors made it impossible to obtain solutions to the simultaneous linear equations established in the variance partitioning matrix.

The remaining option for genetic analysis of the regeneration trait is parent-offspring regression. Regressions of F_3 family mean on F_2 parent for the two crosses are presented in Figure 3-6. Cross 507 shows a strong parent-offspring relationship, with a moderately regression coefficient, or heritability. The coefficient of determination (r^2) is higher for the raw data than for the uncensored data, but the slope is greater for the uncensored data. In the case of cross 501, the regression is not significant. One reason for the poor regression observed for cross 501 is the very small sample size, especially the low number of parents with regeneration scores higher than one. Another reason is the broad range of mean offspring regeneration scores for parents with regeneration scores of one. It is likely that the "true" regeneration scores for some of the parents (particularly the parent at the far lower left of the plot) lie somewhat below the censoring threshold. If it were possible to measure parental regeneration scores of less than one, the observations at the lower left corner of the plot might be distributed farther to the left, resulting in a stronger regression.

The previous discussion of both the callus growth and regeneration traits contained the implicit assumption that the traits were quantitatively inherited. For any genetic system there exists a continuum of possibilities, ranging from

absolute control by a single gene, through control by one or a few major genes in conjunction with a number of minor, or "modifier" genes, to classical quantitative inheritance involving control by many genes with approximately equal effects. The large error variances and non-ideal data structure of this study make it impossible to formally distinguish among these possibilities. However, several general observations and considerations combine to suggest that both of the traits examined are controlled at least to some degree by multiple genes.

First, both traits exhibited transgressive segregation. Callus weights of many F_3 individuals in both crosses greatly exceeded that of any parental individual, and four F_3 individuals from cross 507 received regeneration scores of seven—a score never achieved by the regenerating parent (Figure 3-7; Appendix D). Transgressive segregation in crosses between inbred lines can be attributable to either overdominance or the presence of positive-acting alleles at different loci in each parent. True overdominance is so rare that many authorities doubt its existence (Simmonds, 1979). If single-gene overdominance existed for the regeneration trait, approximately one quarter of F_2 individuals would carry the overdominant genotype, and some proportion of these would likely exhibit the overdominant (transgressive) phenotype. The transgressive phenotype was not observed in the F_2. Thus, there appear to be at least two loci controlling the regeneration trait. In view of the moderately large size of the F_2 populations (57 for cross 501 and 80 for cross 507), the absence of the transgressive phenotype in this generation suggests that more than two loci may be involved.

A second indication that at least two loci control the regeneration trait is the large difference in regeneration scores between Crosses 501 and 507, despite

the fact that the crosses share a regenerating female parent. If ability to regenerate were conferred entirely by alleles originating from one or two loci in the regenerating parent, it would be expected that the best regenerators among the F_2 and F_3 would be those bearing the fixed genotype of the regenerating parent. The best regenerators from each cross would then be expected to perform approximately equally, regardless of the identity of the nonregenerating parent. This was not observed. Instead, the best regenerators from cross 507 consistently outperformed those of cross 501 in both the F_2 and F_3. The transgressive phenotype occurred only in cross 507, suggesting that the nonregenerating parent in this cross contributes at least one positive-acting allele. The issue of quantitative versus qualitative inheritance could be clarified by culturing of a large number of progeny from several high-regenerating F_3 plants.

Some final insights can be obtained by examining the relationship between regeneration and callus growth. Figure 3-8 presents a scattergram of regeneration score versus logarithm of callus weight for the combined F_3 population. A very weak, but significant correlation exists, and perhaps as meaningful is the observation that the high regeneration scores are generally associated with moderate callus growth. This apparent clustering is in part an illusion stemming from the smaller population sizes for the higher regeneration scores. However, there is a significant (α=0.05) decrease in population variances for the callus growth trait as regeneration score increases, indicating that the clustering is real. The association can be either causal, accidental, or both. In both crosses, callus growth was greater in the nonregenerating parent. The absence of regeneration in the high growth calli may therefore be the result of genetic linkage between the two traits—an accident of the distribution of genetic material among the parents. The lack of regeneration among low

growth calli cannot be attributed to linkage. A likely explanation is that a certain degree of in vitro vigor, as manifested by at least a moderate callus growth rate, promotes regeneration. This would constitute pleiotropy in a broad sense. An alternative possibility is that there exist genes that act in a physiologically pleiotropic manner; that is, there may be genes that either promote or inhibit specific metabolic pathways that are necessary for both callus growth and regeneration. To experimentally distinguish between these subtly different hypotheses is far beyond the scope of the present study, or of any study undertaken to date on the genetics of in vitro traits.

Table 3-1. Number of F_2 plants, F_3 plants, and F_3 families from which in vitro data were collected.

Cross	F_2 plants	F_3 plants	F_3 families
501	65	181	11
507	91	291	18
510	52	†	†

† Cross 510 was discontinued after F_2.

Table 3-2. Univariate statistics for callus weight for parental, F_2, and F_3 populations in linear, logarithmic and square root scales.

Population	Scale†	Mean	Variance	Skewness	W	Prob<W	n
144 (Parental)	li	0.7442	0.0648	0.247	0.953	0.288	26
	lo	2.844	0.0268	-0.702	0.934	0.102	26
	sq	26.87	22.89	-0.196	0.985	0.372	26
6123 (Parental)	li	0.2897	0.0172	0.38	0.947	0.119	35
	lo	2.413	0.0477	-0.528	0.95	0.15	35
	sq	16.57	15.44	-0.021	0.964	0.369	35
13083 (Parental)	li	0.7215	0.0541	-0.114	0.958	0.514	20
	lo	2.833	0.0261	-0.93	0.906	0.055	20
	sq	26.49	20.74	-0.503	0.943	0.293	20
501 (F_2)	li	0.301	0.071	2.111	0.794	<0.001	65
	lo	2.319	0.162	-0.432	0.954	0.038	65
	sq	15.93	47.88	0.766	0.942	0.008	65
507 (F_2)	li	0.366	0.069	1.506	0.877	<0.001	92
	lo	2.445	0.123	-0.65	0.9953	0.009	92
	sq	17.97	43.58	0.431	0.969	0.15	92

† li = linear (grams); lo = $\log_{10}$(milligrams); sq = square root (milligrams)

Table 3-2 continued.

Population	Scale†	Mean	Variance	Skewness	W	Prob<W	n
501 (F_3)	li	0.380	0.1138	1.729	0.830	<0.001	181
	lo	2.412	0.1643	-0.242	0.9612	0.001	181
	sq	17.81	63.47	0.691	0.940	<0.001	181
507 (F_3)	li	0.254	0.0526	1.509	0.817	<0.001	291
	lo	2.231	0.1647	-0.161	0.963	<0.001	291
	sq	14.49	43.72	0.737	0.924	<0.001	291

† li = linear (grams); lo = $\log_{10}$(milligrams); sq = square root (milligrams)

Table 3-3. Model coefficients used to construct joint scaling tests.

Population	Coefficient		
	m	[d]	[h]
Parent 1	1	-1	0
Parent 2	1	1	0
F_2	1	0	0.5
F_3	1	0	0.25

Table 3-4. Parameter estimates and associated probabilities obtained from joint scaling tests for callus growth.

Cross	Scale	Parameter							
		m		[d]		[h]		Whole Model	
		estimate	prob>F	estimate	prob>F	estimate	prob>F	χ^2	prob>χ^2
501	Linear	0.497	0.023	0.210	0.057	-0.417	0.091	0.481	0.45
507	Linear	0.401	0.247	0.149	0.567	-0.364	0.637	53.97	<0.001
501	Logarithmic	2.611	0.009	0.210	0.112	-0.672	0.139	2.086	0.24
507	Logarithmic	2.542	0.053	0.228	0.504	-0.582	0.601	89.17	<0.001
501	Square root	21.26	0.020	4.849	0.091	-11.73	0.128	1.360	0.36
507	Square root	19.20	0.148	4.313	0.555	-9.679	0.655	81.66	<0.001

Table 3-5. Coefficients used for variance partitioning for callus growth.

Cross	Observational Component	Causal Component			df
		D	H	E_W	
501	V_1F_2	0.5	0.25	1	64
	V_1F_3	0.5164	0.07076	0.1314	10
	V_2F_3	0.25	0.125	1	170
	W_1F_{23}	0.5	0.125	0	10
	E_1	0	0	1	53
507	V_1F_2	0.5	0.25	1	91
	V_1F_3	0.5196	0.07233	0.1571	17
	V_2F_3	0.25	0.125	1	273
	W_1F_{23}	0.5	0.125	0	17
	E_1	0	0	1	59

Table 3-6. Observational variance components and initial weighs used for variance partitioning for callus growth.

Cross	Scale	Component	Variance	Weight	df
501	Linear	V_1F_2	0.07076	12780	64
		V_1F_3	0.06100	2688	10
		V_2F_3	0.06232	43780	170
		W_2F_{23}	0.08426	582.6	10
		E_1	0.03040	57370	53
501	Logarithmic	V_1F_2	0.1618	2445	64
		V_1F_3	0.09271	1163	10
		V_2F_3	0.08591	23030	170
		W_2F_{23}	0.05925	813.8	10
		E_1	0.03994	33210	53
501	Square Root	V_1F_2	4788	0.02791	64
		V_1F_3	36.46	0.007521	10
		V_2F_3	32.86	0.1575	170
		W_2F_{23}	36.93	0.002687	10
		E_1	17.37	0.1757	53

Table 3-6. Continued.

Cross	Scale	Component	Variance	Weight	df
507	Linear	V_1F_2	0.06941	19100	91
		V_1F_3	0.02204	3500	17
		V_2F_3	0.03618	208600	273
		W_2F_{23}	0.02148	13890	17
		E_1	0.03746	42040	59
507	Logarithmic	V_1F_2	0.1227	6044	91
		V_1F_3	0.06896	3574	17
		V_2F_3	0.1132	21300	273
		W_2F_{23}	0.04864	2612	17
		E_1	0.03879	39210	59
507	Square Root	V_1F_2	43.72	0.04760	91
		V_1F_3	19.19	0.04642	17
		V_2F_3	29.39	0.3160	273
		W_2F_{23}	15.64	0.02542	17
		E_1	18.23	0.1701	59

Table 3-7. Estimates of causal variance components and associated probabilities obtained from variance partitioning for callus growth.

Cross	Scale	Component							
		D		H		E_w		Whole Model	
		Estimate	Prob>F	Estimate	Prob>F	Estimate	Prob>F	F	Prob>F
501	Linear	0.1258	0.4033	-0.0578	0.8239	0.0324	0.1428	28.57	0.1365
507	Linear	0.0313	0.6534	0.0271	0.9055	0.0284	0.2982	11.50	0.2127
501	Logarithmic	0.1273	0.4244	0.1535	0.6266	0.3881	0.1241	48.08	0.1055
507	Logarithmic	0.0594	0.7487	0.3036	0.5601	0.0438	0.2364	11.64	0.2114
501	Square Root	70.39	0.0592	-17.50	0.4434	17.38	0.0189	1944	0.0189
507	Square Root	23.61	0.2057	46.27	0.2916	18.24	0.0512	332.2	0.0403

Table 3-8. Univariate statistics for regeneration score utilizing raw and uncensored data.

Population	Raw Data		Uncensored Data			
	Mean	Variance	Mean	Variance	N	N>1
6123	4.053	1.240	4.075	1.080	38	37
501 F_2 (all)	1.868	1.076	1.679	1.774	57	29
507 F_2 (all)	1.994	1.952	1.278	4.351	80	43
501 F_2 (parents only)	1.636	0.8552	2.078	0.4886	11	4
507 F_2 (parents only)	2.267	2.924	1.553	6.529	17	8
501 F_3	1.594	0.8158	1.185	1.882	165	67
507 F_3	2.058	1.807	1.554	3.733	260	136
501 F_3 families:						
501-1	2.000	1.166	2.116	1.143	13	7
501-2	1.462	0.4359	1.520	0.533	13	6
501-3	1.333	0.4334	1.391	0.7310	21	5
501-4	2.083	1.356	1.982	1.948	12	7
501-5	2.417	2.629	2.045	3.069	12	8
501-6	1.440	0.5067	1.391	0.832	25	8
501-7	1.600	0.7115	1.612	0.986	10	5
501-8	1.214	0.1813	—	—	14	3
501-9	1.923	0.4102	2.032	0.3152	13	11
501-10	1.2143	0.1813	—	—	14	3
501-11	1.389	0.7222	-0.0665	4.920	18	4

Table 3-8. Continued.

Population	Raw Data		Uncensored Data			
	Mean	Variance	Mean	Variance	N	N>1
507 F_3 Families:						
507-1	1.571	0.8791	0.9004	2.974	14	6
507-2	1.769	2.190	—	—	13	3
507-3	1.500	0.3330	—	—	4	2
507-4	3.850	2.976	3.700	2.036	20	20
507-5	3.706	2.220	3.667	1.672	17	16
507-6	1.667	1.466	—	—	6	2
507-7	1.545	0.6406	1.635	0.7582	22	8
507-8	2.000	1.999	1.319	5.197	13	6
507-9	1.500	0.5770	1.438	0.953	14	5
507-10	1.273	0.4182	—	—	11	2
507-11	2.733	2.065	2.641	2.577	15	12
507-12	1.650	0.5553	1.753	0.5090	20	10
507-13	2.625	0.8383	2.625	0.6933	8	7
507-14	1.714	1.214	1.220	2.965	21	8
507-15	2.000	0.9091	2.356	1.188	12	7
507-16	1.611	0.6050	1.680	0.668	18	8
507-17	1.789	1.397	1.056	4.177	19	8
507-18	1.615	0.7564	1.386	1.365	13	6

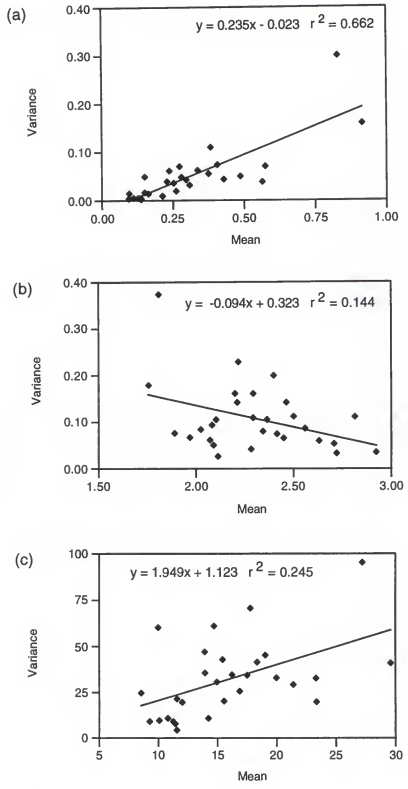

Figure 3-1. Relationship of F_3 family means and variances for callus growth under (a) linear, (b) logarithmic, and (c) square root scales.

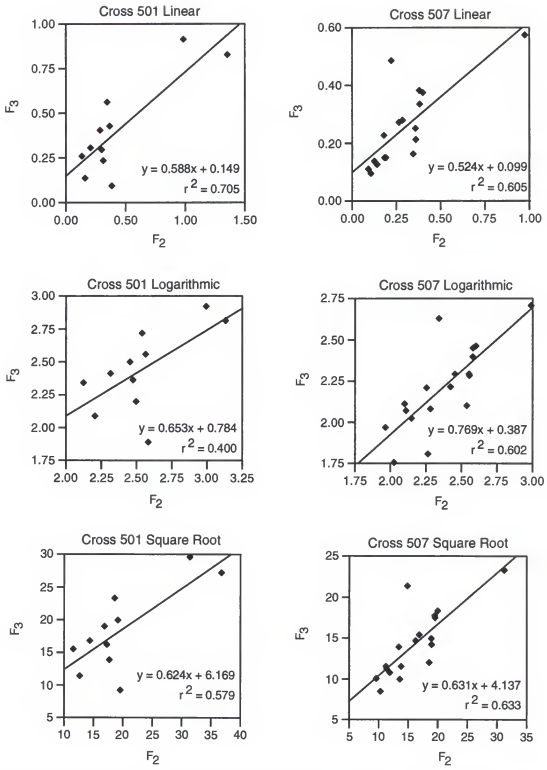

Figure 3-2. Regression of F_3 family mean callus weight on F_2 parental callus weight under linear, logarithmic, and square root scales.

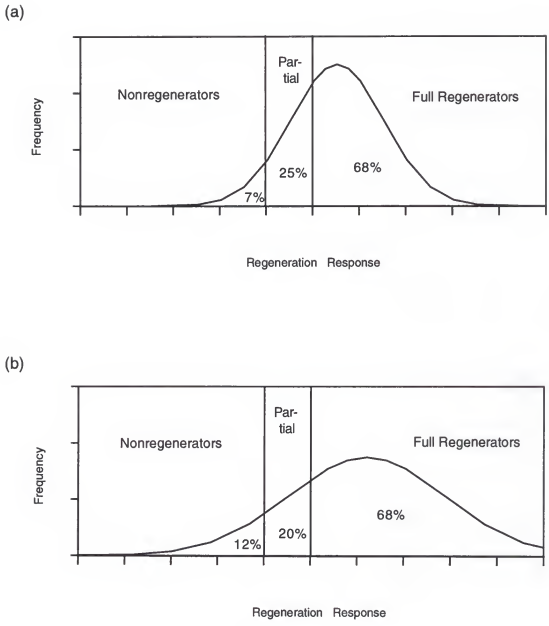

Figure 3-3. Two hypothetical distributions with three response classes and two thresholds.

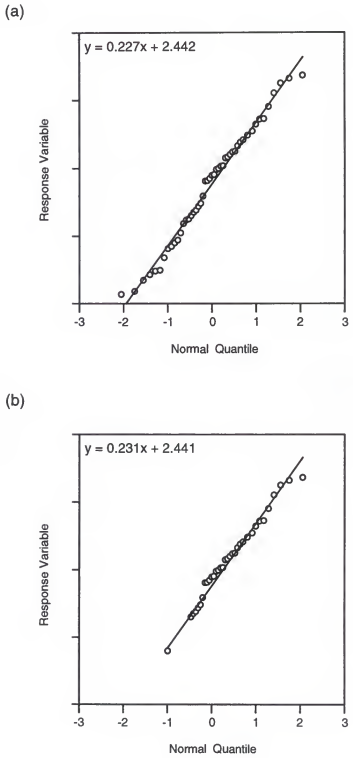

Figure 3-4. Normal quantile plots of a hypothetical (a) intact and (b) censored population.

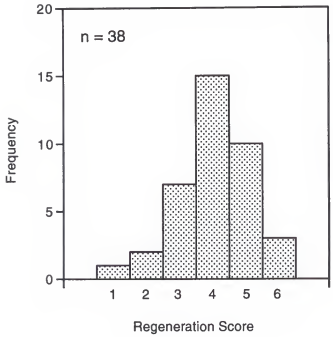

Figure 3-5. Regeneration score histogram for genotype IRFL 6123.

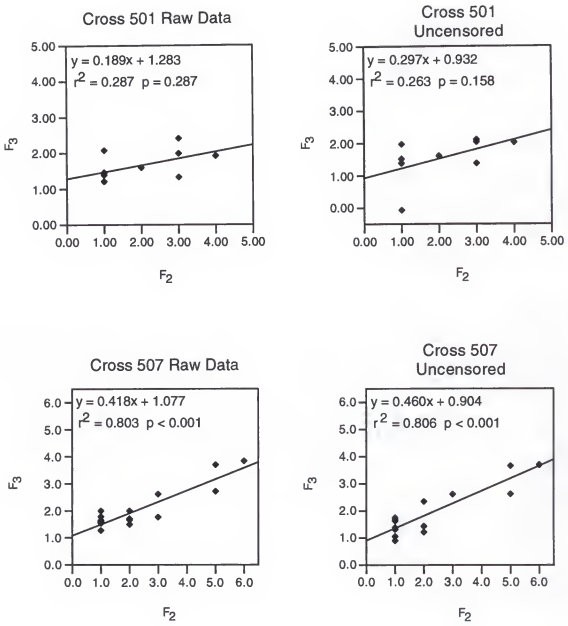

Figure 3-6. Regression of F_3 family mean regeneration score on F_2 parent regeneration score.

Figure 3-7. Profuse regeneration exhibited by members of two F_3 families derived from cross 507

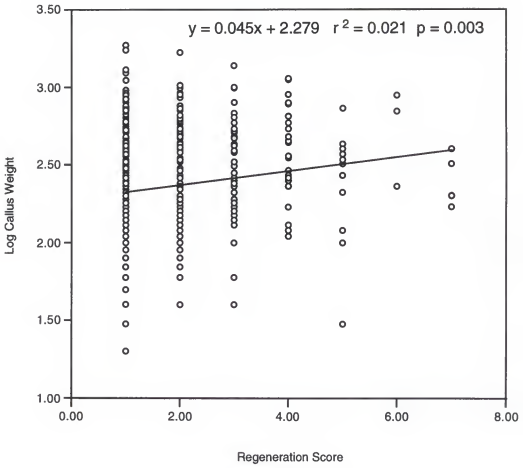

Figure 3-8. Regression of callus weight on regeneration score for combined F_3 populations.

CHAPTER 4
SUMMARY AND CONCLUSIONS

The point of departure for this dissertation was a study conducted by Wofford et al. (1992b). Wofford's study identified a three stage (callus induction, shoot induction, and shoot elongation) protocol utilizing the L2-based media of Phillips and Collins (1979) as suitable for tissue culture of desmodium hypocotyls, and obtained limited regeneration from a single genotype—IRFL 6123. The objective of the current work has been to examine the potential for enhancing in vitro response in desmodium through improvements in culture protocol and through breeding.

Chapter 2 focused on optimization of culture protocols. The work was not intended to be an exhaustive investigation of potential methods for enhancing in vitro response in desmodium. Given the wide range of variables that can determine the effectiveness of tissue culture protocols and the limited prior work with this crop, such an investigation would be beyond the scope of any single study. Instead, the intent was to cast a broad net—to determine if the poor, highly genotype-specific regeneration observed in desmodium could be significantly improved by applying a wide range of techniques similar to those that had proven successful in other legumes. Accordingly, within each general culture strategy (e.g., alternative explant sources, manipulation of auxin-cytokinin ratio, substitution of different auxin sources) only a small number of specific culture protocols were examined. For the same reason, number of replications was often fairly low. Thus, failure, for example, to obtain regeneration from leaf disks or petioles does not indicate that regeneration from

these explant types can not be achieved—only that none of the methods tested yielded strongly encouraging results.

The first general culture strategy investigated was the use of alternative explant sources. In addition to the previously proven hypocotyl explants, leaf disks, petioles, and seedling cotyledons were tested. One previously established regenerating genotype (IRFL 6123) and two nonregenerating genotypes (CIAT 13083 and UF 20) were tested. Regeneration was not obtained from any of the alternative explant sources under the single culture protocol tested. The only repeatable regeneration response was from hypocotyls of IRFL 6123, as had been previously reported (Wofford et al., 1992b).

Two more strategies for broadening the range of regenerating genotypes were examined. The first was manipulation of 2,4-D concentration, with and without added kinetin, in the shoot induction medium. This experiment failed to induce regeneration in recalcitrant genotypes, but succeeded in identifying an optimal 2,4-D concentration for regeneration in IRFL 6123. In addition, kinetin was found to inhibit regeneration in this genotype. The last attempt at inducing regeneration in recalcitrant genotypes involved direct regeneration from hypocotyl explants. This approach failed to yield regeneration even from IRFL 6123.

The remainder of Chapter 2 dealt with enhancing the regeneration response in IRFL 6123. The first experiment demonstrated that reducing the shoot induction period from the original 28 days to 14 days had no effect on regeneration. Two additional experiments investigated the effects of growth regulators in the shoot elongation step. An optimum ratio of 0.012 mg L^{-1} picloram to 0.2 mg L BA was identified.

Histological examination of regenerating cultures revealed vascular connections between shoots and the surrounding callus tissue, indicating that regeneration was of an organogenic nature. However, early in the shoot induction period, small structures resembling somatic embryos could occasionally be observed. The combination of growth regulators used in the shoot induction step is similar to that used for somatic embryogenesis in other species, so it is possible that the regeneration observed in this study was some aberrant form of somatic embryogenesis.

It was clearly established in Chapter 2 that regeneration in desmodium is highly dependent on genotype and that broadening the range of regenerating genotypes through modification of culture protocols is difficult or impossible. Chapter 3 focused on the genetics of callus growth and regeneration. An extensive effort at crossing regenerating with nonregenerating genotypes yielded only three crosses—501, 507, and 510. Cross 510 proved useless for the analysis at hand due to severe internodal stunting and other morphological abnormalities in the F_1 and F_2 generations. This may have been the result of cytoplasmic effects, since cross 510 was essentially the reciprocal of cross 507, which showed no abnormalities. Examination of the genetics and physiology of this phenomenon might produce very interesting results.

The remaining two crosses were selfed through the F_3 generation. Analysis of callus growth and and regeneration in these crosses produced many ambiguous results, but also yielded useful insights. A large amount of effort was devoted to scaling of the callus growth trait. General statistical considerations indicated that a simple linear scale was inadequate for describing this trait. Logarithmic and square root transformation were both more satisfactory than the linear scale, but it was not clear which of these transformations was superior.

Formal genetic analysis of callus growth was approached via the joint scaling test and variance partitioning. Neither method yielded conclusive results. This may have been due to violation of the genetic assumptions inherent in both methods, or may have been because both methods inappropriately attempted to describe the trait with a simple additive-dominant genetic model. Parent offspring regression yielded heritabilities ranging from 0.52 to 0.77, depending on cross and scale. It appears, then, that callus growth in this germplasm is controlled to a significant extent by additive genetic effects, but that there may in addition be nonadditive, nondominant genetic effects that act to confound the joint scaling test and variance partitioning.

The regeneration trait presented unusual analytical difficulties. Many calli showed no evidence of regeneration. As a result the majority of population distributions were severely truncated. Truncated (or "censored") distributions cannot be rendered normal by any mathematical transformation. A procedure was described by which truncated population distributions can be reconstructed, or "uncensored." While this procedure has apparently not been previously applied to genetic analysis, it is conceptually similar to the threshold trait approach described by Falconer (1981). The method is less than ideal, but is preferable to attempting to analyze raw, severely censored data. Constraints imposed by the data structure and by the uncensoring technique made it impossible to conduct meaningful joint scaling tests or variance partitioning analyses. Parent-offspring regression yielded relatively high heritability estimates for cross 507—0.416 for raw data; 0.460 for uncensored data. No significant regression was obtained for cross 501. It appears likely that this failure is due to shortcomings of the data structure, and that regeneration is in fact weakly to moderately heritable in this cross.

Vasil (1987) has suggested that the genetics of regeneration is irrelevant, and that with sufficient insight into the in vitro physiology of a species regeneration can be induced in even the most difficult genotype. While this may be true in theory, this work suggests that such an assumption can be impractical or even counterproductive. Approximately equal effort was directed at culture protocol and genetic approaches and the latter was found to be by far the more productive path. It is noteworthy that the original regenerating parent regenerated under a variety of culture conditions, suggesting a general genetic proclivity to regenerate. A more experienced investigator with greater resources may or may not have obtained better results from culture protocol optimization, but success may well have been very costly in terms of time, effort, and financial resources. The genetic approach has been relatively straightforward and strikingly successful. The magnitude of this success was particularly apparent from visual examination of regenerating calli. Several F_3 calli, including that shown in Figure 3-7, grossly outperformed the parental genotype, continuing to produce vigorous shoots through repeated subcultures, and ultimately yielding dozens of shoots.

This study has demonstrated that regeneration in desmodium can be greatly improved by conventional crossing techniques. Limited light was shed on the genetic basis of regeneration in this crop, and many questions remain unanswered. It is hoped that the F_3 material produced in this study may prove useful to any investigator who might desire to perform regeneration-dependent work with this crop.

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APPENDIX A
ANALYSIS OF VARIANCE FOR BETWEEN- AND WITHIN-FAMILY VARIANCES FOR CALLUS GROWTH

Between- and within-family variances were estimated by analysis of variance with F_3 family as the class variable. All ANOVA analyses yielded highly significant results. Expected mean square for families equals $(\sigma_w^2 + n\sigma_b^2)$, where σ_w^2 is within-family variance (estimated by MSE), n is harmonic mean of number of individuals per family, and σ_b^2 is between-family variance. This equation is solved for $\sigma_b^2 = (MS_{model} - MSE)/n$. The following table presents MS_{model}, MSE, σ_b^2, σ_w^2, and n for each cross and each scale.

Cross	Scale	MS_{model}	$MSE(= \sigma_w^2)$	σ_b^2	n
501	Linear	0.9901	0.0623	0.0669	15.21
501	Logarithmic	1.4962	0.0859	0.9271	"
501	Square Root	583.95	32.856	36.462	"
507	Linear	0.3166	0.0361	0.0220	12.73
507	Logarithmic	0.9910	0.1132	0.0689	"
507	Square Root	273.78	29.392	19.199	"

APPENDIX B
DERIVATION OF VARIANCE PARTITIONING MATRICES FOR CALLUS GROWTH

Coefficients for variance partitioning of the callus growth were based on those given by Mather and Jinks (1971, 1977). The following general coefficients were given for the populations treated in the present study:

Observational Component	D	H	E_W	E_B	Sampling Variance
V_{1F2}	1/2	1/4	1	0	0
V_{1F3}	1/2	1/16	0	1	1/n V_{2F3}
V_{2F3}	1/4	1/8	1	0	0
W_{1F23}	1/2	1/8	0	0	0
E_1	0	0	1	0	0

Since there is only one entry for sampling variance and the sampling variance for V_{1F3} is a function of V_{2F3}, this column can be combined into the other columns. In the case of cross 501, n (the harmonic mean of the number of individuals per F_3 family) is equal to 15.21. Therefore, after combining the sampling variance column, the coefficient for D for V_{1F3} becomes $1/2 + ((1/15.21) \times 1/4)$, or 0.516435. The other entries in the V_{1F3} row are similarly altered. Since E_B is a function of E_W, this column can also be

eliminated. The coefficient for E_w for V_{1F3} becomes the sum of the contribution to this column from the V_{2F3} row (1/15.21 x 1) plus the contribution from the E_b column in the V_{1F3} row (1/15.21), or 0.1314. The resulting table is shown below:

	D	H	E_W
V_{1F2}	1/2	1/4	1
V_{1F3}	0.516435	0.0707175	0.1314803
V_{2F3}	1/4	1/8	1
W_{1F23}	1/2	1/8	0
E_1	0	0	1

Derivation of coefficients for cross 507 are similar, but n for this cross is 12.73.

APPENDIX C
RESULTS OF SECOND AND THIRD ITERATIONS OF VARIANCE PARTITIONING FOR CALLUS GROWTH

Results of variance partitioning second iteration:

Cross	Scale	Component							
		D		H		E_w		Whole Model	
		Estimate	Prob>F	Estimate	Prob>F	Estimate	Prob>F	F	Prob>F
501	Linear	0.1362	0.3918	-0.0672	0.8024	0.0334	0.1578	27.73	0.1385
507	Linear	0.0289	0.7176	0.0411	0.8616	0.0138	0.2744	9.16	0.2372
501	Logarithmic	0.1327	0.3790	0.1501	0.6365	0.4253	0.1218	49.48	0.1040
507	Logarithmic	0.0637	0.7024	0.3337	0.5073	0.0445	0.2465	14.89	0.1877
501	Square Root	70.02	0.0642	-18.68	0.4518	17.37	0.0206	1641	0.0181
507	Square Root	23.89	0.1992	46.39	0.2828	18.21	0.0503	335.7	0.0401

Results of variance partitioning third iteration:

Cross	Scale	Component							
		D		H		E_w		Whole Model	
		Estimate	Prob>F	Estimate	Prob>F	Estimate	Prob>F	F	Prob>F
501	Linear	0.1372	0.4024	-0.0692	0.8090	0.0323	0.1528	28.57	0.1365
507	Square Root	23.90	0.1999	46.37	0.2830	18.21	0.0500	336.9	0.0400

APPENDIX D
RAW CALLUS GROWTH AND REGENERATION DATA

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
507-1	1	1	1	80	129
507-1	2	1	1	140	129
507-1	3	1	1	220	129
507-1	4	1	1	100	129
507-1	5	1	1	40	129
507-1	6	1	1	140	129
507-1	7	1	1	40	129
507-1	8	2	1	290	129
507-1	9	2	1	90	129
507-1	10	4	1	120	129
507-1	11	1	1	130	129
507-1	12	1	1	130	129
507-1	13	2	1	180	129
507-1	14	3	1	190	129
507-2	1	1	3	130	265
507-2	2	4	3	440	265
507-2	3	1	3	370	265
507-2	4	1	3	290	265
507-2	5	4	3	350	265
507-2	6	1	3	850	265
507-2	7	1	3	710	265
507-2	8	1	3	60	265
507-2	9	1	3	110	265
507-2	10	1	3	70	265
507-2	11	1	3	40	265
507-2	12	5	3	30	265
507-2	13	1	3	90	265
507-3	1	2	2	70	191
507-3	2	2	2	340	191
507-3	3	1	2	90	191
507-3	4	1	2	100	191
507-4	1	3	6	180	180
507-4	2	3	6	40	180
507-4	4	2	6	340	180
507-4	5	2	6	90	180
507-4	6	3	6	190	180
507-4	7	2	6	760	180
507-4	8	4	6	250	180
507-4	9	7	6	170	180

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
507-4	10	5	6	100	180
507-4	11	3	6	60	180
507-4	13	2	6	470	180
507-4	14	7	6	200	180
507-4	15	5	6	730	180
507-4	16	2	6	140	180
507-4	19	5	6	430	180
507-4	20	4	6	110	180
507-4	21	3	6	150	180
507-4	22	7	6	320	180
507-4	23	3	6	210	180
507-4	24	5	6	270	180
507-5	1	1	5	590	975
507-5	2	6	5	890	975
507-5	3	3	5	510	975
507-5	4	3	5	430	975
507-5	5	4	5	450	975
507-5	6	4	5	650	975
507-5	7	3	5	480	975
507-5	8	4	5	1120	975
507-5	11	4	5	290	975
507-5	14	3	5	430	975
507-5	15	6	5	700	975
507-5	16	2	5	140	975
507-5	17	3	5	990	975
507-5	18	7	5	400	975
507-5	19	3	5	230	975
507-5	20	4	5	780	975
507-5	21	3	5	680	975
507-6	1	1	2	20	184
507-6	2	1	2	20	184
507-6	3	2	2	100	184
507-6	4	1	2	20	184
507-6	5	1	2	150	184
507-6	6	4	2	590	184
507-7	1	3	1	310	285
507-7	2	1	1	170	285
507-7	3	3	1	470	285
507-7	4	1	1	150	285
507-7	8	3	1	380	285
507-7	9	2	1	150	285
507-7	10	1	1	580	285
507-7	11	1	1	640	285
507-7	12	1	1	130	285
507-7	14	2	1	660	285
507-7	15	1	1	50	285

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
507-7	16	1	1	330	285
507-7	17	1	1	230	285
507-7	18	1	1	110	285
507-7	19	1	1	240	285
507-7	20	3	1	800	285
507-7	21	2	1	110	285
507-7	22	1	1	530	285
507-7	23	1	1	280	285
507-7	24	1	1	300	285
507-7	25	2	1	150	285
507-7	26	1	1	90	285
507-8	1	1	1	960	400
507-8	2	2	1	590	400
507-8	3	1	1	130	400
507-8	4	1	1	440	400
507-8	6	1	1	440	400
507-8	7	1	1	400	400
507-8	8	5	1	400	400
507-8	9	4	1	360	400
507-8	10	2	1	200	400
507-8	11	1	1	220	400
507-8	12	2	1	490	400
507-8	13	4	1	120	400
507-8	14	1	1	470	400
507-9	1	3	1	420	380
507-9	2	1	1	140	380
507-9	3	1	1	520	380
507-9	4	1	1	390	380
507-9	5	1	1	910	380
507-9	6	1	1	850	380
507-9	8	3	1	130	380
507-9	9	2	1	900	380
507-9	10	1	1	250	380
507-9	13	1	1	1110	380
507-9	14	1	1	200	380
507-9	15	2	1	400	380
507-9	16	1	1	170	380
507-9	19	2	1	180	380
507-10	2	1	1	140	106
507-10	3	2	1	80	106
507-10	4	3	1	380	106
507-10	5	1	1	360	106
507-10	8	1	1	20	106
507-10	9	1	1	40	106
507-10	10	1	1	20	106

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
507-10	11	1	1	20	106
507-10	12	1	1	30	106
507-10	13	1	1	70	106
507-10	14	1	1	50	106
507-11	1	2	5	160	360
507-11	2	1	5	120	360
507-11	3	1	5	110	360
507-11	4	5	5	320	360
507-11	5	3	5	260	360
507-11	6	5	5	370	360
507-11	7	2	5	200	360
507-11	8	3	5	180	360
507-11	9	2	5	80	360
507-11	10	1	5	130	360
507-11	11	5	5	210	360
507-11	12	3	5	250	360
507-11	13	2	5	380	360
507-11	14	2	5	130	360
507-11	15	4	5	290	360
507-12	1	1	1	190	92
507-12	2	3	1	260	92
507-12	3	1	1	90	92
507-12	5	1	1	80	92
507-12	6	1	1	110	92
507-12	8	1	1	340	92
507-12	10	1	1	140	92
507-12	11	1	1	100	92
507-12	13	1	1	120	92
507-12	14	2	1	120	92
507-12	16	2	1	160	92
507-12	18	1	1	100	92
507-12	20	2	1	70	92
507-12	21	3	1	170	92
507-12	22	2	1	60	92
507-12	23	2	1	80	92
507-12	24	1	1	50	92
507-12	25	3	1	100	92
507-12	26	2	1	140	92
507-13	1	4	2	480	382
507-13	2	3	2	210	382
507-13	3	2	2	270	382
507-13	4	2	2	290	382
507-13	5	2	2	220	382
507-13	6	2	2	170	382
507-13	7	4	2	900	382
507-13	8	2	2	150	382

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
507-14	1	3	2	640	220
507-14	2	1	2	160	220
507-14	3	1	2	630	220
507-14	4	2	2	120	220
507-14	5	1	2	540	220
507-14	6	1	2	730	220
507-14	7	1	2	800	220
507-14	8	1	2	720	220
507-14	9	1	2	580	220
507-14	10	4	2	270	220
507-14	11	1	2	420	220
507-14	12	1	2	380	220
507-14	13	1	2	250	220
507-14	14	4	2	650	220
507-14	15	1	2	170	220
507-14	16	2	2	580	220
507-14	17	4	2	540	220
507-14	18	1	2	360	220
507-14	19	2	2	330	220
507-14	20	1	2	920	220
507-14	21	2	2	420	220
507-15	1	3	1	240	141
507-15	3	2	1	240	141
507-15	5	2	1	120	141
507-15	6	1	1	70	141
507-15	7	1	1	200	141
507-15	8	1	1	200	141
507-15	9	3	1	130	141
507-15	10	1	1	150	141
507-15	11	3	1	130	141
507-15	12	3	1	140	141
507-15	14	1	1	70	141
507-15	15	3	1	100	141
507-16	1	2	1	160	126
507-16	2	1	1	60	126
507-16	3	1	1	130	126
507-16	4	3	1	160	126
507-16	5	2	1	180	126
507-16	6	3	1	210	126
507-16	7	1	1	190	126
507-16	8	1	1	120	126
507-16	9	2	1	110	126
507-16	10	1	1	210	126
507-16	11	1	1	160	126
507-16	12	1	1	70	126
507-16	13	1	1	90	126

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
507-16	14	1	1	90	126
507-16	15	2	1	120	126
507-16	16	1	1	150	126
507-16	17	2	1	90	126
507-16	18	3	1	180	126
507-17	2	3	2	380	344
507-17	3	1	2	370	344
507-17	4	3	2	180	344
507-17	5	5	2	340	344
507-17	7	1	2	150	344
507-17	8	1	2	90	344
507-17	10	1	2	40	344
507-17	11	4	2	130	344
507-17	12	1	2	100	344
507-17	13	2	2	200	344
507-17	14	2	2	430	344
507-17	15	2	2	40	344
507-17	17	1	2	240	344
507-17	19	2	2	120	344
507-17	20	1	2	120	344
507-17	21	1	2	70	344
507-17	22	1	2	250	344
507-17	23	1	2	230	344
507-17	24	1	2	70	344
507-18	1	2	1	300	358
507-18	2	1	1	60	358
507-18	3	2	1	340	358
507-18	4	2	1	130	358
507-18	5	1	1	150	358
507-18	6	1	1	380	358
507-18	7	1	1	90	358
507-18	8	2	1	190	358
507-18	9	1	1	320	358
507-18	10	4	1	790	358
507-18	11	1	1	340	358
507-18	12	2	1	190	358
507-18	13	1	1	200	358
501-1	1	1	3	100	160
501-1	2	2	3	120	160
501-1	3	3	3	150	160
501-1	4	1	3	150	160
501-1	5	1	3	120	160
501-1	7	1	3	200	160
501-1	8	3	3	210	160
501-1	9	3	3	280	160
501-1	10	4	3	170	160
501-1	11	1	3	70	160

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
501-1	12	2	3	70	160
501-1	13	3	3	160	160
501-1	14	1	3	90	160
501-2	1	1	1	1240	984
501-2	2	1	1	1110	984
501-2	3	2	1	860	984
501-2	4	3	1	1000	984
501-2	5	1	1	890	984
501-2	6	2	1	600	984
501-2	7	1	1	1300	984
501-2	8	1	1	740	984
501-2	9	2	1	350	984
501-2	10	1	1	750	984
501-2	11	1	1	1870	984
501-2	12	1	1	550	984
501-2	13	2	1	620	984
501-3	1	1	3	210	298
501-3	2	1	3	320	298
501-3	3	1	3	420	298
501-3	4	1	3	370	298
501-3	5	2	3	440	298
501-3	6	3	3	140	298
501-3	7	2	3	90	298
501-3	8	2	3	610	298
501-3	9	1	3	190	298
501-3	10	1	3	130	298
501-3	13	1	3	310	298
501-3	14	1	3	130	298
501-3	15	1	3	560	298
501-3	16	1	3	330	298
501-3	17	1	3	510	298
501-3	18	1	3	130	298
501-3	19	1	3	830	298
501-3	20	1	3	130	298
501-3	21	3	3	160	298
501-3	22	1	3	150	298
501-3	23	1	3	530	298
501-4	1	3	1	590	1352
501-4	2	2	1	710	1352
501-4	3	1	1	190	1352
501-4	4	1	1	580	1352
501-4	5	2	1	920	1352
501-4	6	4	1	1140	1352
501-4	7	2	1	1680	1352
501-4	8	1	1	240	1352
501-4	9	1	1	540	1352

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
501-4	10	1	1	1740	1352
501-4	11	3	1	1380	1352
501-4	12	4	1	230	1352
501-5	3	1	3	80	382
501-5	7	6	3	230	382
501-5	8	3	3	170	382
501-5	9	2	3	130	382
501-5	10	1	3	110	382
501-5	11	2	3	150	382
501-5	12	2	3	230	382
501-5	13	1	3	50	382
501-5	17	5	3	120	382
501-5	18	2	3	90	382
501-5	20	3	3	130	382
501-5	21	1	3	100	382
501-6	1	1	1	360	284
501-6	2	1	1	200	284
501-6	3	1	1	130	284
501-6	4	2	1	120	284
501-6	5	1	1	110	284
501-6	6	2	1	220	284
501-6	7	1	1	380	284
501-6	8	1	1	610	284
501-6	9	1	1	320	284
501-6	10	3	1	520	284
501-6	11	1	1	240	284
501-6	12	1	1	530	284
501-6	13	1	1	360	284
501-6	14	1	1	150	284
501-6	15	2	1	990	284
501-6	16	1	1	290	284
501-6	18	2	1	1030	284
501-6	19	1	1	760	284
501-6	20	1	1	830	284
501-6	22	1	1	480	284
501-6	23	1	1	130	284
501-6	24	2	1	500	284
501-6	25	3	1	240	284
501-6	27	3	1	390	284
501-6	28	1	1	300	284
501-7	2	3	2	180	313
501-7	3	1	2	180	313
501-7	4	1	2	80	313
501-7	5	1	2	50	313
501-7	6	3	2	330	313
501-7	7	1	2	40	313

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
501-7	8	2	2	90	313
501-7	9	1	2	140	313
501-7	10	1	2	410	313
501-7	11	2	2	900	313
501-8	1	1	1	130	206
501-8	2	1	1	330	206
501-8	3	1	1	250	206
501-8	4	2	1	340	206
501-8	5	1	1	90	206
501-8	6	1	1	390	206
501-8	7	2	1	220	206
501-8	8	1	1	670	206
501-8	9	1	1	90	206
501-8	10	1	1	190	206
501-8	11	1	1	210	206
501-8	12	1	1	360	206
501-8	13	2	1	610	206
501-8	14	1	1	430	206
501-9	1	2	4	550	365
501-9	2	1	4	390	365
501-9	3	3	4	160	365
501-9	4	2	4	130	365
501-9	5	2	4	660	365
501-9	6	1	4	90	365
501-9	7	2	4	430	365
501-9	8	2	4	520	365
501-9	9	3	4	540	365
501-9	10	1	4	480	365
501-9	11	2	4	260	365
501-9	12	2	4	630	365
501-9	13	2	4	730	365
501-10	1	1	1	700	345
501-10	2	1	1	190	345
501-10	3	2	1	290	345
501-10	4	1	1	830	345
501-10	5	1	1	420	345
501-10	6	1	1	480	345
501-10	7	1	1	720	345
501-10	8	1	1	590	345
501-10	9	1	1	600	345
501-10	10	2	1	410	345
501-10	11	2	1	660	345
501-10	12	1	1	840	345
501-10	13	1	1	450	345
501-10	14	1	1	710	345
501-11	1	1	1	240	133

Family	F_3 Individual	Regeneration Score		Callus Weight	
		Progeny	Parent	Progeny	Parent
501-11	2	2	1	480	133
501-11	3	4	1	260	133
501-11	4	3	1	140	133
501-11	5	1	1	80	133
501-11	7	1	1	140	133
501-11	8	1	1	440	133
501-11	9	1	1	270	133
501-11	10	1	1	200	133
501-11	11	2	1	540	133
501-11	12	1	1	420	133
501-11	13	1	1	270	133
501-11	14	1	1	330	133
501-11	15	1	1	370	133
501-11	16	1	1	180	133
501-11	17	1	1	260	133
501-11	18	1	1	120	133
501-11	19	1	1	180	133

BIOGRAPHICAL SKETCH

Peter Krottje was born in Haskell, New Jersey, in 1951. He attended Fordham University, receiving a B.S. in biology in 1973. He received an M.S. in soil science from the University of Florida in 1980. He has been very fortunate.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1995

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